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14. ABSTRACT
The overall goal of this research project is to develop and implement a conceptually innovative strategy for down-regulating nuclear receptors such as the androgen receptor: the creation of bifunctional molecules that simultaneously bind to the nuclear receptor and to chromatin-modifying enzymes and/or complexes. In this way, genes that are typically regulated by androgen receptor and glucocorticoid receptor will be extrinsically controlled. In this final year of funding, we completed the functional characterization of agonist and antagonist-based bi-functional recruiters. Importantly, we have a suite of recruiters that show context specificity. In the extension period of the work, we completed RNAseq experiments to gain a complete understanding of the context specificity of this exciting class of molecules.

15. SUBJECT TERMS

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INTRODUCTION

Androgen receptor (AR) is an allosterically regulated transcription factor that binds to both the endogenous steroids testosterone and 5α-dihydrotestosterone and to a range of synthetic ligands. AR is central to prostate cancer pathogenesis and its reactivation is a hallmark of castration-resistant prostate cancer (CRPC), an aggressive and terminal illness for which there is no effective treatment. Despite this disease progression, most androgen-refractory prostate cancers continue to rely on AR for their survival; thus it remains an important therapeutic target.[1] Historically, approaches to modulate AR have focused on targeting the ligand-binding pocket with small molecules that sculpt the surface of the receptor in unique ways.[2] This indirect remodeling of the receptor binding surface results in the recruitment of different native binding partners. Although a powerful strategy, it has already been found that mutation of either binding surface (i.e. AR or cofactor) can occur, leading to, for example, antagonists that later become agonists. Even with the development of second-generation anti-androgens and small molecules that target sites other than the ligand-binding domain of AR, new small molecules are urgently needed that can suppress AR function and do not rely on allostery to elicit their effect.[3,4,5,6] In this project, we have developed an innovative alternative strategy to specifically steer and extend the repertoire of receptor-coregulator partnerships. By targeting ligand and substrate pockets in the receptor and coregulator complexes, we proposed to bridge the two using novel small molecule bifunctional recruiters (Figure 1).

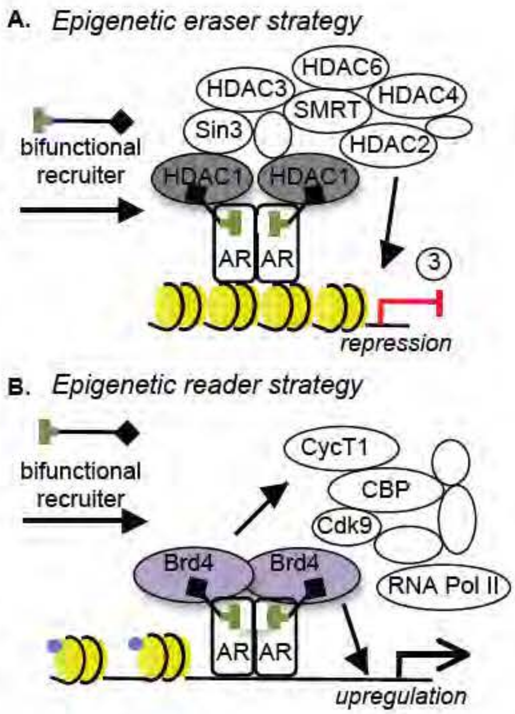


Figure 1. A. Epigenetic eraser strategy to block AR function. Bifunctional recruiters contain a high affinity AR ligand and an isoform-selective HDAC inhibitor (HDACi) can recruiting corepressor complexes to AR. One functional consequence of this targeted recruitment could be deacetylation of chromatin, repressing transcription. **B.** Epigenetic reader strategy to enhance AR function. Bi-functional recruiters contain a high affinity bromodomain inhibitor to recruit Brd4 to AR regulated promoters. One functional consequence of this mode of targeted recruitment could be up-regulation, as depicted. However, transcriptional inhibition may be observed due to steric blockade at some genes.

Our original experimental plan focused upon one of the best-characterized mechanisms of transcriptional inhibition, the recruitment of large corepressor complexes that harbor histone deacetylase (HDAC) activity (Figure 1A).[7,8,9,10,11,12] By appropriately linking HDAC inhibitors to high-affinity nuclear receptor ligands, we proposed to generate a new class of molecules that recruit transcriptionally repressing complexes to AR, a predicted consequence being the suppression of AR genomic function. As outlined in more detail in the body of this progress report, the targeted bi-functional molecules performed in vitro as designed and in cellular model systems; the preliminary studies supporting our model were published last year and, importantly, were chosen as the 'Best of Basic Research 2014' by the Endocrine Society (http://press.endocrine.org/bestofbasicresearch/2014) (Appendix I). However, in cellular models of prostate cancer, no significant gene-specific or phenotypic effects were observed. We thus implemented the alternative strategy outlined in the original proposed work plan and illustrated in Figure 1B. In this strategy, we use the potent bromodomain inhibitor JQ1 to recruit Brd4 and thus extrinsically alter the transcriptional status of the targeted genes. As shown in the second funding period (months 13-24) this strategy was successfully implemented in a full-length nuclear receptor mode, albeit not in an endogenous setting. In this final funding period we focused on three goals: (1) demonstration that altered transcriptional response is due to recruitment accomplished by the bifunctional molecule (Task 4); (2) assessment of agonist and

antagonist-based bifunctional recruiters in the context of endogenous genes; (3) genome-wide assessment of the best candidates via RNAseq in order to guide the design of the next generation of molecules. Because this has taken considerable additional effort that originally outlined in the Statement of Work, we requested and were granted a no-cost extension.

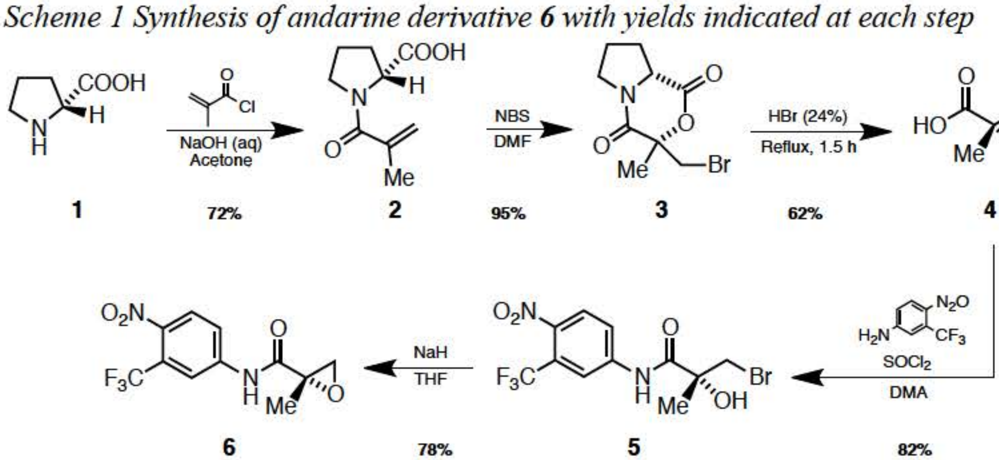
BODY

Summary of ***Task 1*** *goals:* The primary focus of Task 1 was the design and synthesis of bi-functional molecules consisting of high-affinity AR ligands linked to class-selective HDACi. Full in vitro characterization of the bi-functional molecules comprises this Task. Molecules that demonstrated affinity for both targets comparable to unmodified inhibitors (within 2- to 4-fold) were carried on to *Task 2*.

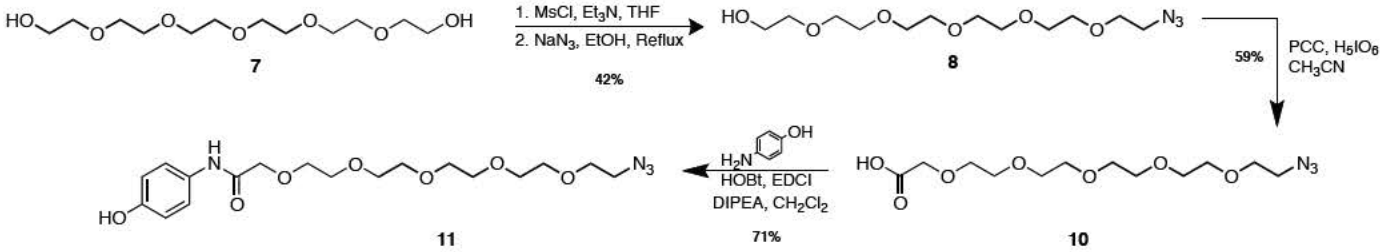
Accomplishments of ***Task 1***

As outlined in the Year One and Year Two progress reports, the goals of Task 1 were largely accomplished in the first twelve months of the Project. Briefly, the syntheses of two AR-targeted bifunctional molecules were completed and their binding abilities characterized. In both cases, the AR-targeting agent andarine was used, as it is a well-characterized partial agonist of AR. In addition, a PEG-derived linker was used to connect the two components, based upon an initial feasibility study that we completed using the closely related nuclear receptor GR as the target; the details of this study are found in Appendix 1. The HDAC inhibitor components of the bifunctional molecules were that of SAHA, a pan-specific HDACi, and the class I HDAC inhibitor, PD106. PD106 was a particularly attractive inhibitor choice because it is a slow, tight-binding inhibitor of Class 1 HDACs and also forms an especially stable complex with HDAC3.[13] SAHA is an excellent comparison HDACi because it inhibits more broadly across HDAC classes and has different binding kinetics. The synthesis of the individual components of the target as well as the bifunctional molecule itself are outlined in Schemes 1, 2 and 3. Although not explicitly shown in these schemes, the PD106 analog (**PD106-alkyne**) needed for conjugation was also synthesized and characterized for this purpose. Our synthetic route to these bifunctional molecules is highly modular. For example, **Andarine-O5-N3** is an advanced intermediate that can be coupled to a variety of HDACi. The results obtained from our initial studies with these inhibitors informed the synthesis of the next generation of ligands, taking advantage of our key intermediate; this strategy was used, for example, to synthesize the bifunctional molecules that target Brd4 developed alter in the project.

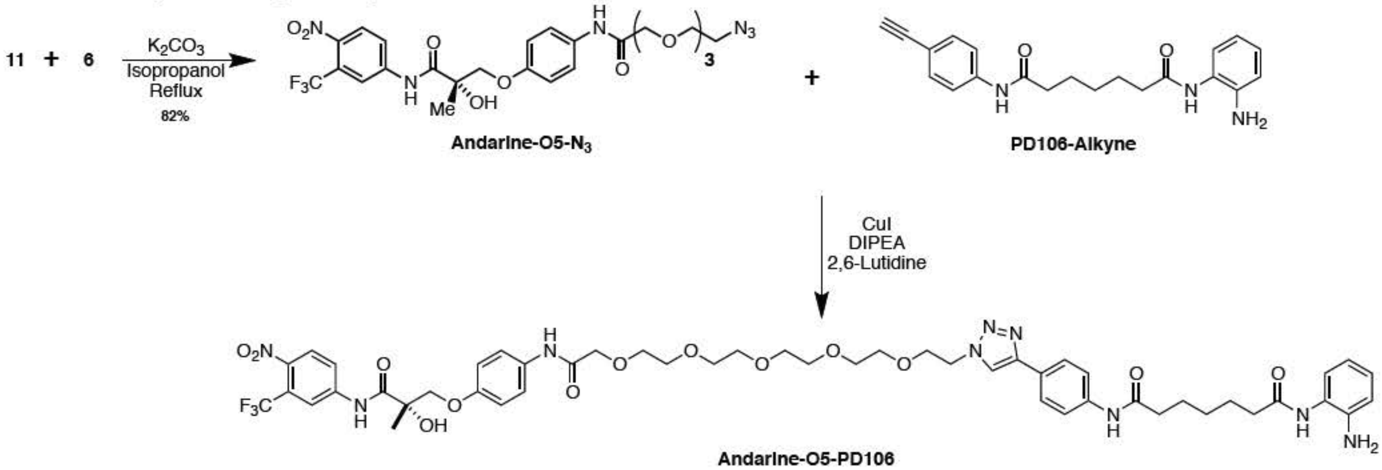
Scheme 1 Synthesis of andarine derivative ***6*** *with yields indicated at each step*



*Scheme 2: Synthesis of the hydrophilic linker suitable for conjugation (**11**)*



*Scheme 3: Synthesis of the bifunctional molecule **andarine-O5-PD106***



As outlined in the Statement of Work and in the body of the original proposal, a critical assessment at this stage was how the binding of each component of the bifunctional molecule to either AR or the HDAC(s) in question was impacted by conjugation. As measured by a Fluor-de-Lys assay and illustrated below, both SAHA and PD106 remain effective HDAC inhibitors even after modification. SAHA-derived bifunctional molecules showed only a small (<5-fold) attenuation in K_i for both HDAC1 and HDAC3, consistent with data in the literature.[14,15] For PD106, we examined PD106 itself, a biotinylated conjugate (**biotin-PD106**) and **andarine-05-PD106**. As shown in Figure 3, under our assay conditions, PD106 is a potent, slow-binding inhibitor of HDAC1 and HDAC3. However, upon conjugation to form a bifunctional molecule, the affinity of the PD106 moiety for HDAC1 drops considerably whereas the affinity for HDAC3 is affected to a much smaller degree (approximately 2-fold; Figure 4). Although this result was somewhat surprising, it may prove to be an advantage in future work as the conjugate exhibits increased selectivity for the HDAC3 isoform.

Figure 3. *Affinity of PD106 for HDAC1 and HDAC3*

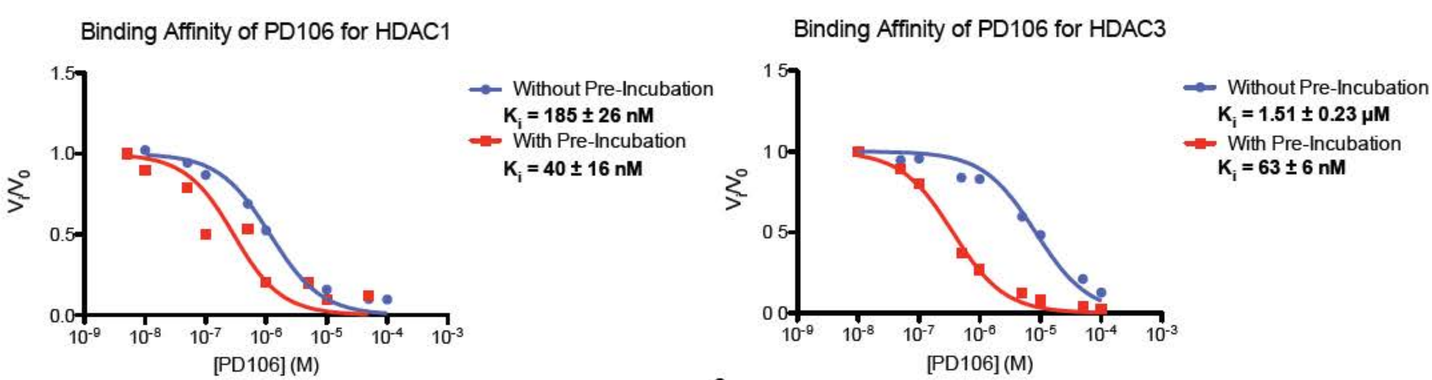
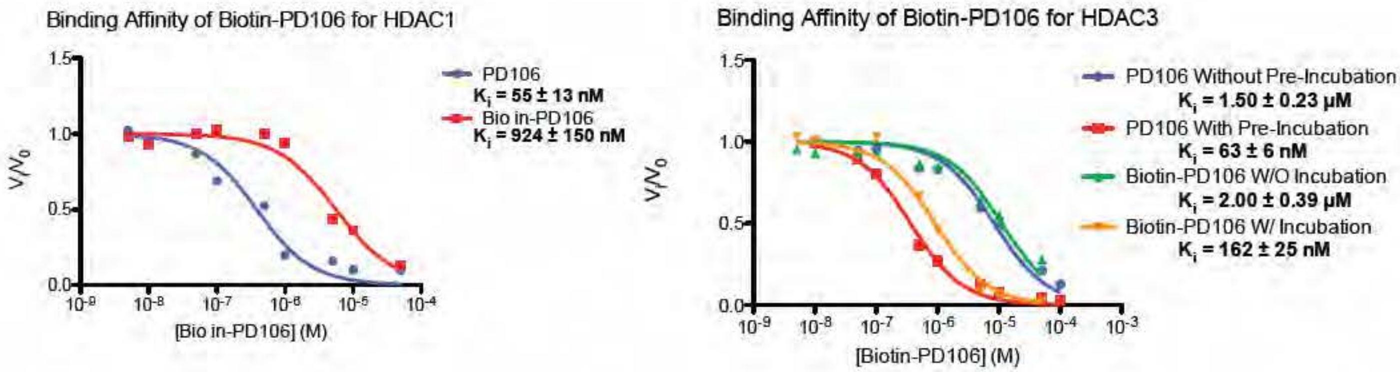
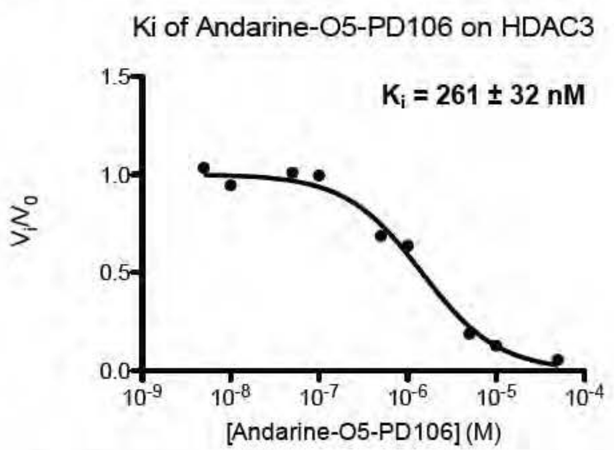


Figure 4. *Conjugation to PD106 affects HDAC1 affinity but not HDAC3 affinity*



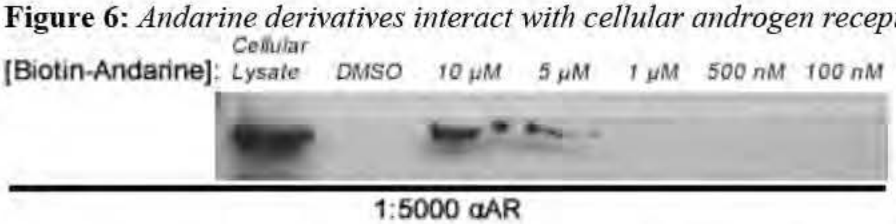
The same experiment was then carried out with **andarine-O5-PD106** and, consistent with the results obtained with the biotinylated version of PD106, the affinity for HDAC3 was affected only 4-fold (Figure 5). **Thus, the andarine-O5-PD106 conjugate met our criteria for advancing to Task 2 with regard to HDAC inhibition.** This was also true of SAHA-derived bifunctional molecules.

Figure 5. ***Andarine-O5-PD106*** is an effective inhibitor of HDAC3



To test if the bifunctional molecules maintained affinity for AR, we carried out pull-down experiments with a biotinylated variant of andarine. Following this procedure, the bifunctional andarine-biotin conjugate was found interact with cellular androgen receptor in a dose-dependent manner (Figure 6). Thus, andarine is a strong candidate for future bi-functional molecule designs.

Figure 6: *Andarine derivatives interact with cellular androgen receptor as monitored by pull-down*



A manuscript including preliminary results from Task 1 was accepted for publication and can be found in Appendix I (Jonas W. Højfeldt, Osvaldo Cruz-Rodríguez, Yasuhiro Imaeda, Aaron R. Van Dyke, James P. Carolan, Anna K. Mapp and Jorge A. Iñiguez-Lluhi, *Molecular Endocrinology* **2014** *28* 249-59).

*Summary of **Task 2** goals:* Bifunctional molecules developed in the previous task will be examined in different cell lines (PC-3, LNCaP, VCaP) for their ability to modulate exogenous (luciferase) and endogenous (PSA, ERG) AR-driven reporters. Control experiments will also be performed to compare the effects of bifunctional molecules and HDACi alone (i.e. HDACi without an AR-targeting moiety). Bifunctional recruiters that demonstrate cellular activity will be further examined as outlined in *Task 3.* If bi-functional molecules do not show efficacy in these assays, replacement of the HDACi recruitment moiety with an alternative inhibitor of chromatin modifying activities will be examined.

*Accomplishments of **Task 2***

Three-hybrid assay to assess recruitment by bi-functional molecules

The data from Aim 1 established that the bi-functional ligand design attenuated the affinity of each component ligand to its respective targets by only 2-5-fold. In this set of experiments, the goal was to assess if this would translate to recruitment of both targets to a promoter through a three-hybrid assay. In this experimental setup, a fusion of the HDAC of interest (HDAC3 is shown) to VP16 is expressed in the cells. As shown in Figure 7, if the bi-functional molecule interacts with AR and with HDAC3 then the potent VP16 activation domain should lead to transcriptional activation of a luciferase reporter gene or an endogenous gene.

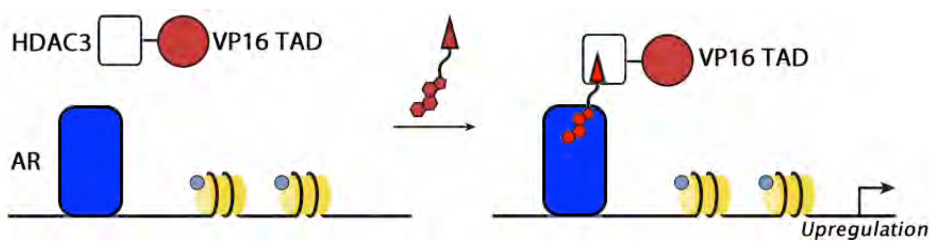
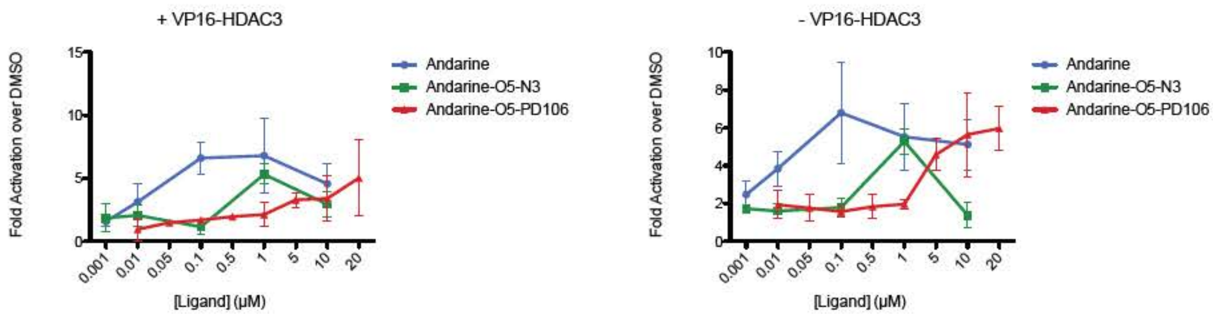


Figure 7. Schematic of 3-hybrid assay used to test bi-functional molecules. In this assay the the HDAC of interest is fused to the potent transcriptional activator VP16; as an example, an HDAC3-VP16 construct is illustrated. Thus, if the bifunctional molecule interacts with both AR and the targeted HDAC at the promoter, then transcriptional up-regulation will be observed.

Andarine-O5-PD106 and hAR ± VP16-HDAC3

HEK293T cells were transfected with plasmids encoding full-length androgen receptor (1 μg), a luciferase reporter containing the prostate specific antigen promoter (1 μg), a fusion protein of HDAC3 with the VP16 TAD at its N-terminus and a FLAG tag at the C-terminus (1 μg), and a CMV driven β-galactosidase reporter (40 ng) using Lipofectamine 2000 (10 μL) in Optimem media. Additionally, a second aliquot of cells were transfected using the same cocktail with the exception of the HDAC3 fusion protein, which was replaced with pBSSK+ to maintain the total amount of transfected DNA between experiments. The cells were allowed to recover overnight following transfection and were then trypsinized and plated on a 96 well plate (20,000 cells per well). After adhering for eight hours, cells were dosed with various concentrations of Andarine, Andarine-O5-N_3, Andarine-O5-PD106, or DMSO as a vehicle control for eighteen hours. The cells were then lysed and the luciferase and β-galactosidase activity were assayed. Activation of the PSA reporter plasmid was normalized using β-galactosidase activity and is reported as fold activation over DMSO.

Figure 8. **Results from 3-hybrid experiments with HDAC1-VP16 fusion protein.** Data shown is the average of three independent experiments with the indicated errors (SDOM).



The moderate activation of the reporter plasmid by andarine is consistent with data reported in the literature and confirms that the cells were effectively transfected.[1] Andarine-O5-PD106 is also capable of activating the reporter to a moderate extent, though only at high concentrations and without any dependence on the presence of the VP16 fusion. Thus, the activation observed for the bifunctional molecule is not the result of dimerization of the target proteins, but instead is likely due to the inherent attenuated activation potential of the andarine component of the molecule.

Andarine-O5-PD106 and hAR + HDAC1-VP16

HEK293T cells were transfected as described above with the exception that the plasmid encoding the HDAC3 fusion protein was replaced with a plasmid encoding an HDAC1 fusion protein with the VP16 TAD at its C-terminus. The cells were allowed to recover and dosed as described above before luciferase and β-galactosidase activity were assayed.

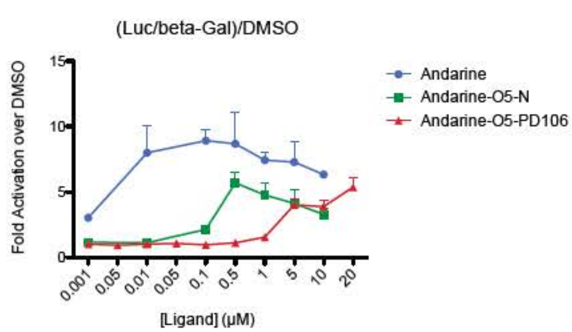
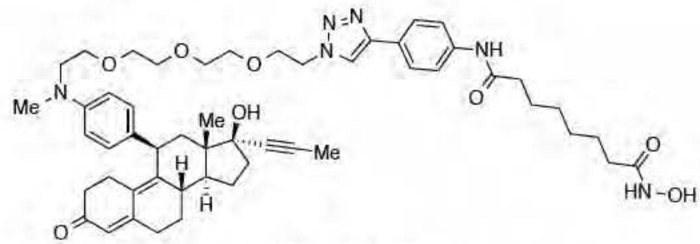


Figure 9. **Results from 3-hybrid experiments with HDAC1-VP16 fusion protein.** Data shown is the average of three independent experiments with the indicated errors (SDOM).

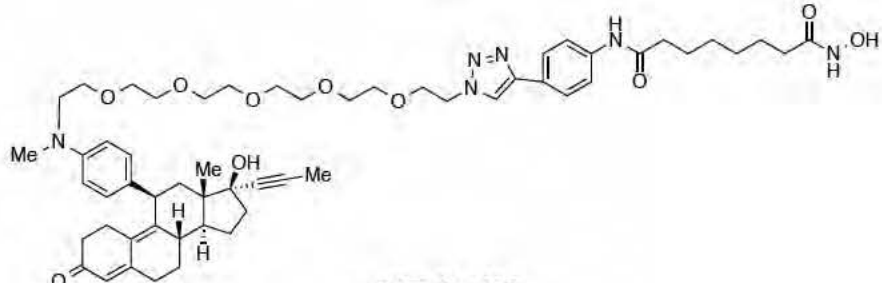
PD106 is a slow, tight-binding Class I (HDAC1, HDAC2, HDAC3, and HDAC8) selective HDAC inhibitor.[13] After the observation that Andarine-O5-PD106 could not dimerize AR and HDAC3, the experiment was repeated using an alternative Class I HDAC, HDAC1 (Figure 9). Dosing with andarine again resulted in moderate activation of the reporter, confirming the cells were effectively transfected. Consistent with the results using VP16-HDAC3, the bifunctional molecule failed to dimerize the target proteins and the observed activation at high concentrations of the conjugate is again likely due to the inherent activation potential of the andarine component of the molecule.

RU486-O3/O5-SAHA and hAR ± VP16-HDAC3

After the disappointing results of the previous experiments we hypothesized that the use of an anti-androgen as the AR targeting agent would lead to an AR form that would be more easily controlled extrinsically. Towards that end, we designed and synthesized two bifunctional molecules composed of the anti-antrogen RU486 conjugated to the pan-specific HDAC inhibitor SAHA through a polyethylene glycol linker.[16,17] Two molecules containing different linker lengths were prepared and assessed to determine the impact of linker length on the activity of the molecules. HEK293T cells were transfected as described for the previously described VP16-HDAC3 three-hybrid experiment. Following the transfection, cells were allowed to recover and were then dosed with various concentrations of RU486-O3-SAHA, RU486-O5-SAHA, RU486-O3-N_3, andarine, or DMSO as a vehicle control for eighteen hours before luciferase and β-galactosidase activity were assayed.



RU486-O3-SAHA



RU486-O5-SAHA

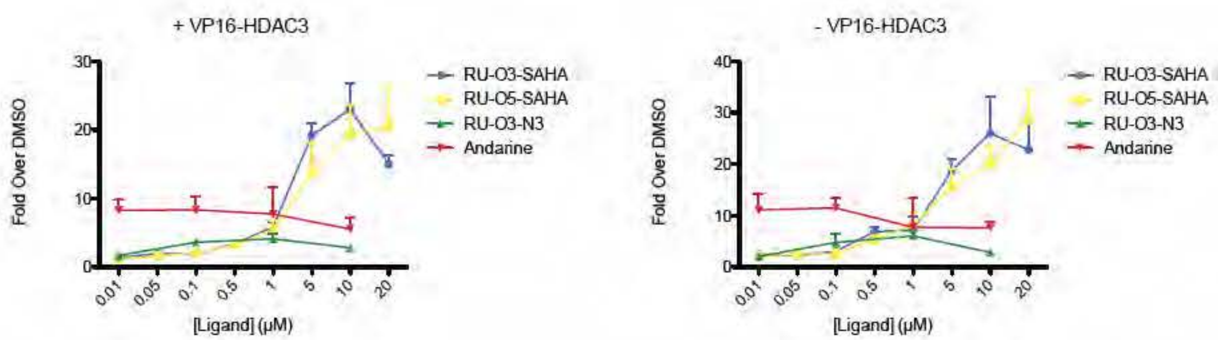
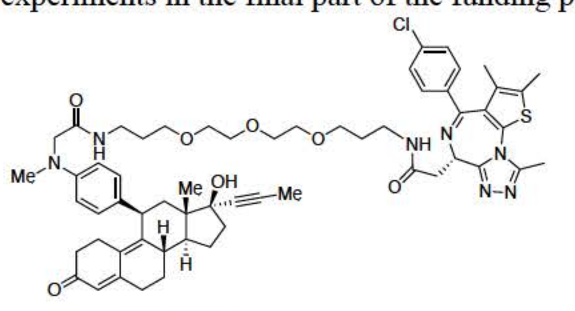


Figure 10. Three-hybrid assessment of RU486-based bifunctional molecules. Above are the structures of the two RU486-based molecules, differing only in the linker length connecting the two functional units. Results are the average of three experiments, with the indicated error (SDOM).

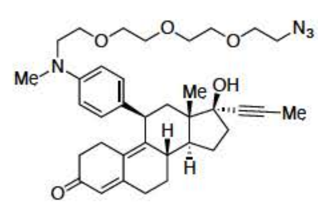
Consistent with previous experiments, andarine led to moderate activation of the reporter, confirming the effective transfection of the cells. Dosing with RU486-O3-N_3 led to very low activation (~1-4 fold over DMSO), consistent with the function of the parent molecule as a partial agonist antiandrogen.[16] The two conjugates tested led to significant activation at high concentrations of small molecule, with no apparent dependence on linker length. Interestingly, this activation is also not dependent upon the presence of the VP16 containing HDAC3 fusion protein. These data suggest that the conjugate is not dimerizing the target proteins with the desired effect, as transcription is significantly potentiated rather than abrogated even in the absence of the VP16 fusion protein.

Several reports suggest that treatment with low concentrations of SAHA can lead to enhanced transcriptional output in exogenous reporter systems.[18,19,20] The loss of transcriptional activity at high doses of the *trans* addition (> 5 μM) is consistent with the reported IC_{50} values for SAHA in cellular proliferation assays against several prostate cancer cell lines.[21] Thus, the activity of the bifunctional molecule is explained by the activities of the individual component molecules, RU486 and SAHA. Furthermore, the conjugate displays lower potency and efficacy than the addition of the molecules *in trans*, which is likely a result of the diminished binding affinity of the proteins for the molecules due to the modifications made in order to link them.

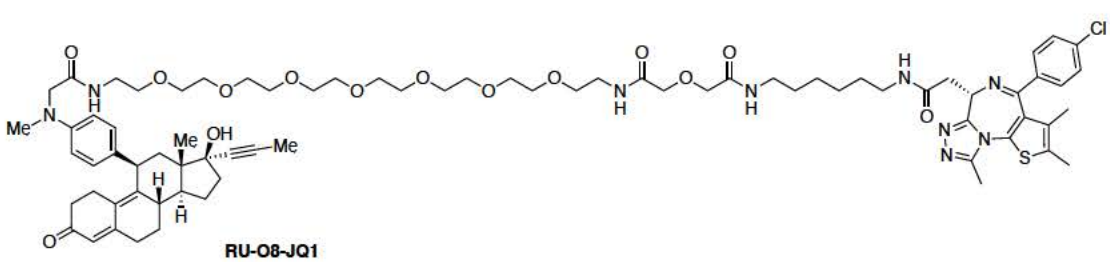
<u>Re-design of bi-functional molecule</u> Our data from Task 1 and results from our model system (Appendix 1) illustrate the fundamental feasibility of extrinsic control of androgen receptor function through bi-functional recruiters. The primary difficult with the HDACi-based molecules appears to be ineffective recruitment at the promoter level. Thus we sought to replace the HDACi moiety with an alternative recruiter and target epigenetic reader proteins (i.e. bromodomains) instead of epigenetic erasers (i.e. HDACs). (S)-JQ1 is a highly potent and selective of bromodomain 4 (BRD4) which is known to intereact with critical components of the transcriptional machinery (e.g. CDK9, cyclin T1, RNA Pol II). Because our bifunctional molecules are constructed modularly, it was straightforward to conjugate existing advanced intermediates with JQ1 using our synthetic strategies developed for Task 1. Additionally, we established a productive collaboration with Professor Jay Bradner and Dr. Jun Qi (the discoverers of JQ1) at Dana Farber Cancer Institute in order to facilitate meaningful functional and mechanistic analysis of our JQ1-based bifunctional molecules. In Year 2, we outlined the successful synthesis and analysis of RU486-based JQ1 conjugates shown below in a reporter gene context and these were carried on to the Task 3 and Task 4 experiments in the final part of the funding period.



RU-O3-JQ1



RU-O3



RU-O8-JQ1

Agonist-based JQ1 bifunctional molecules In addition to the RU-based JQ1 conjugates, in Year 3 we prepared and examined bifunctional constructs based upon dexamethasone. (Figure 11) As outlined in the previous Progress Report, the dynamic range for most androgen receptor-based transcriptional assays is sufficiently small that dissecting structure-function relationships for the bifunctional conjugates is difficult at best. Thus, we continued the use of glucocorticoid receptor (GR) as the model in which to test the bifunctional recruitment model. Given the modular nature of our design, lessons learned with GR will be readily portable to AR.

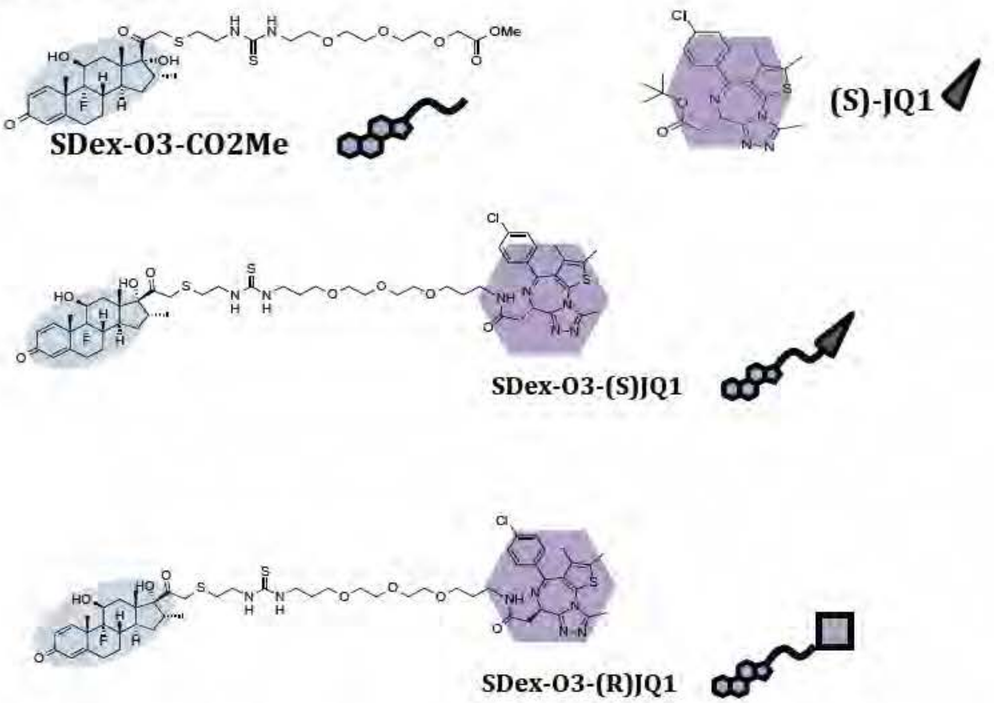


Figure 11 Summary of BRD4-targeting ligands. The ligands used in subsequent studies include unmodified (S)-JQ1, a monofunctional GR ligand-linker compound SDex-O3-CO2Me, a bifunctional SDex-O3-(S)JQ1 , and a bifunctional molecule conjugated to an inactive enantiomer of JQ1 termed SDex-O3-(R)JQ1.

Functional assessment of SDex-JQ1 bifunctional recruiters To test these constructs we have targeted full length human glucocorticoid receptor (hGR) as we and others have observed a more robust dynamic range in the transcriptional response. U2OS cells were transfected with a reporter plasmid bearing a consensus glucocorticoid response element (GRE) immediately upstream of a promoter driving luciferase expression and an expression plasmid coding for human GR. Cells were treated with either SDex-O3-CO2Me or SDex-O3-JQ1 (100 nM) for the indicated time. Transcriptional activity was determined by monitoring luciferase expression and displayed as fold activation over the levels of luciferase in vehicle-treated cells (Fig. 12). Both the monofunctional and bifunctional ligands acted as agonists of GR-mediated transcription, stimulating activity that increased with increasing incubation time. A 16-hour treatment produced the strongest transcriptional response and was chosen for further investigations into the activity of SDex-O3-JQ1.

To examine effects at endogenous promoters, U2OS cells were transfected with luciferase reporter plasmids driven by different GR-regulated promoters and incubated with the indicated compound(s) for 16

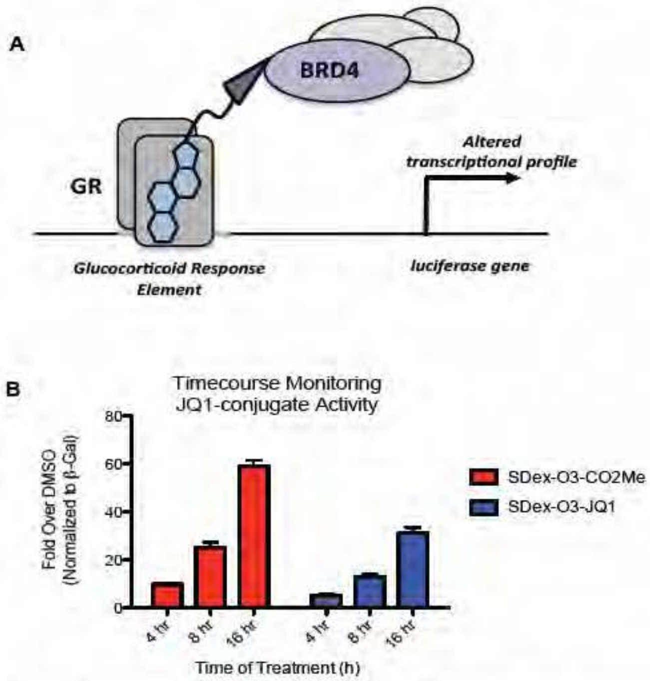


Figure 12 Recruitment of BRD4 to full-length GR. A reporter experiment was designed utilizing full-length GR (A). B: the activity of each GR-ligand was time dependent, with maximal observed activity occurring at 16 hours

hours. This measurement of transcriptional activity is displayed as a relative percentage normalized to the activity produced by dosing with SDex-O3-CO2Me (100 nM) in Figure 13 In addition to doses with the monofunctional SDex-O3-CO2Me and bifunctional SDex-O3-JQ1, a 'trans' addition dosing of equimolar SDex-O3-CO2Me and a biotinylated form of (S)-JQ1 [(S)-JQ1-biotin] and a 'squelch' dosing including SDex-O3-JQ1 and an excess (10 μM) of (S)-JQ1-biotin were included. The trans addition components were chosen to ensure that transcriptional outcomes caused by SDex-O3-JQ1 are not simply additive or synergistic responses provoked independently by each moiety in the bifunctional molecule, while the squelch dosing is included to confirm that effects are induced through BRD4 recruitment. As expected, SDex-O3-CO2Me acts agonistically to activate GR-driven transcription, while co-dosing with (S)-JQ1-biotin results in a suppression of transcription at high (1 μM) concentration, but otherwise minimally affects the activity of SDex-O3-CO2Me. Increasing concentrations of SDex-O3-JQ1 result in a bell-shaped activity curve, initially increasing at low concentration but declining with higher concentrations of compound; at the highest dose (1 μM), activity levels are approximately 50% of the maximum stimulation level (produced at 10 nM). The co-addition of excess (S)-JQ1-biotin increases activity, albeit not to the same level as seen with the trans addition treatment. The suppression of activity seen with SDex-O3-JQ1, paired with the observation that free (S)-JQ1-biotin competes with suppression, suggests that the recruitment of BRD4 is interfering with GR-mediated transcription. **As outlined in Task 4, these and other data indicate that bi-functional recruitment is the driver for the altered gene expression patterns observed, a critical finding for the overall project.** Thus, the SDex-based bifunctional molecules in addition to the RU486-based constructs were carried forward to the Task 3 experiments.

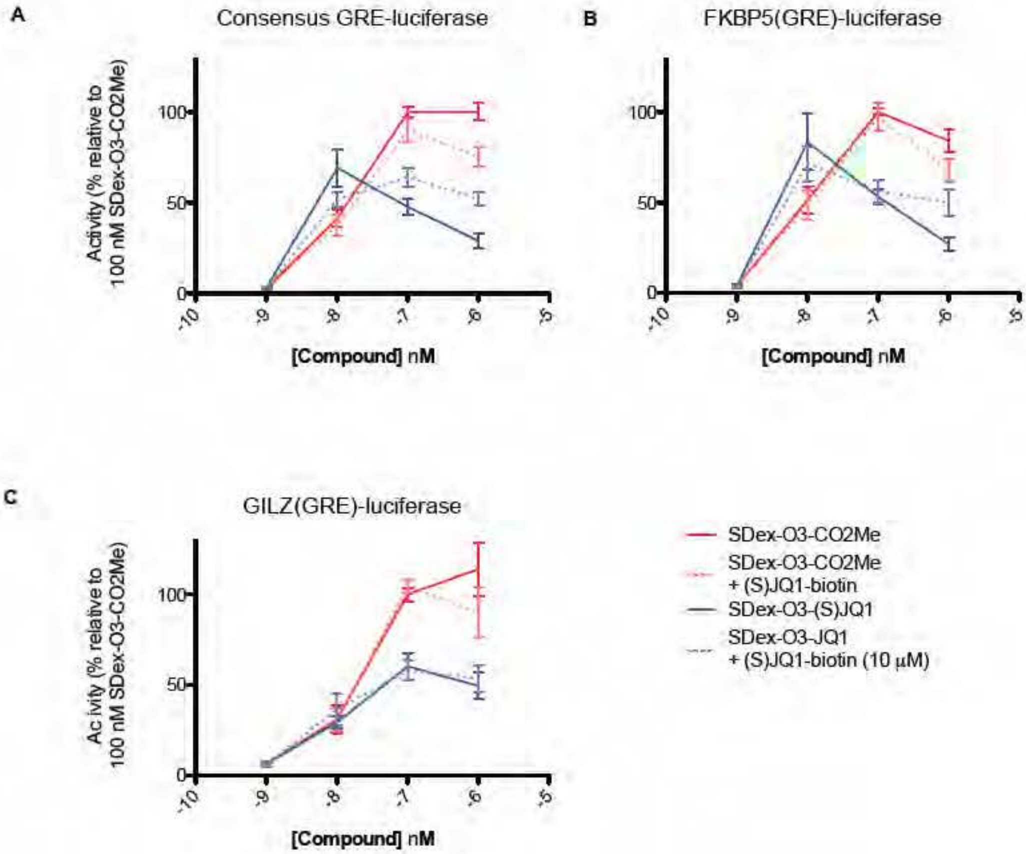


Figure 13 Utilizing alternative GREs in reporter experiments. Full-length GR was utilized in a transcriptional reporter experiment, with a reporter plasmid using a consensus GRE (A), *FKBP5* GRE (B), or *GILZ* GRE (C).

***Summary of* Task 3 goals**. The overall goal of Task 3 is to quantitatively define the unique properties of the bifunctional recruiters as well as assess their mechanism of action. In the original Statement of Work, the subtasks described were centered around HDACi-based recruiters, examining alterations in acetylation patterns, for example. Because we moved on to bromodomain-targeting bifunctional recruiters, the details of the experiments shifted to the definition of the unique properties of these specific molecules. In the Year 2 progress report, we described the characterization of RU486 (antagonist)-based bifunctional recruiters. In months 25-36 we completed an examination of agonist (SDex)-based JQ1 recruiters at a suite of endogenous genes with endogenous full length receptor in comparison with controls and, finally, the genome-wide analysis to determine the context-specificity of the molecules.

Accomplishments of* Task 3 *goals

<u>SDex-JQ1 bifunctional recruiters at endogenous promoters</u> To investigate the effects of bifunctional molecule treatment on endogenous gene expression, U2OS cells were transfected with an expression plasmid for the human GR and treated with the indicated compound(s). Following treatment, cells were lysed, total RNA isolated, and the indicated transcript was quantified relative to the levels in vehicle-treated cells. The transcriptional activity is displayed as fold activation in Fig. 14 The relative transcript levels of *S100P* (Fig. 14A), *FKBP5* (Fig. 14B), and *GILZ* (Fig. 14C) were determined. SDex-O3-CO2Me treatment activated the transcription of each these three GR-target genes. Unlike in reporter experiments, co-dosing with (S)-JQ1-biotin did not appreciably alter the activity of SDex-O3-CO2Me; this may be a byproduct of a

shorter dosing time in RNA quantification studies, though the transcriptional response to both SDex and (S)-JQ1 is rapid. In comparison to SDex-O3-CO2Me, SDex-O3-JQ1 weakly activated transcription of the *S100P* gene, raising levels to approximately 25% of the maximum level induced by SDex-O3-CO2Me. Consistent with the hypothesis that BRD4 recruitment inhibits GR activity, the addition of excess (S)-JQ1 allows SDex-O3-JQ1 to activate *S100P* transcription to similar levels as SDex-O3-CO2Me. SDex-O3-JQ1 induced a similar, but less pronounced, effect in activating transcription of the *FKBP5* gene to approximately 60% of the level induced by SDex-O3-CO2Me (Fig. 14B). Again, addition of excess (S)-JQ1 squelches the suppressed agonism by SDex-O3-JQ1, raising activity to a level comparable with SDex-O3-CO2Me. Interestingly, the transcriptional response at the *GILZ* gene was even more disparate; treatment with SDex-O3-JQ1 alone or in tandem with (S)-JQ1 produced a near identical activation of *GILZ* transcription. While further analysis of additional targets is necessary prior to making conclusions, the wide range in activity induced by the bifunctional glucocorticoid points to the context specificity of the bifunctional recruiters.

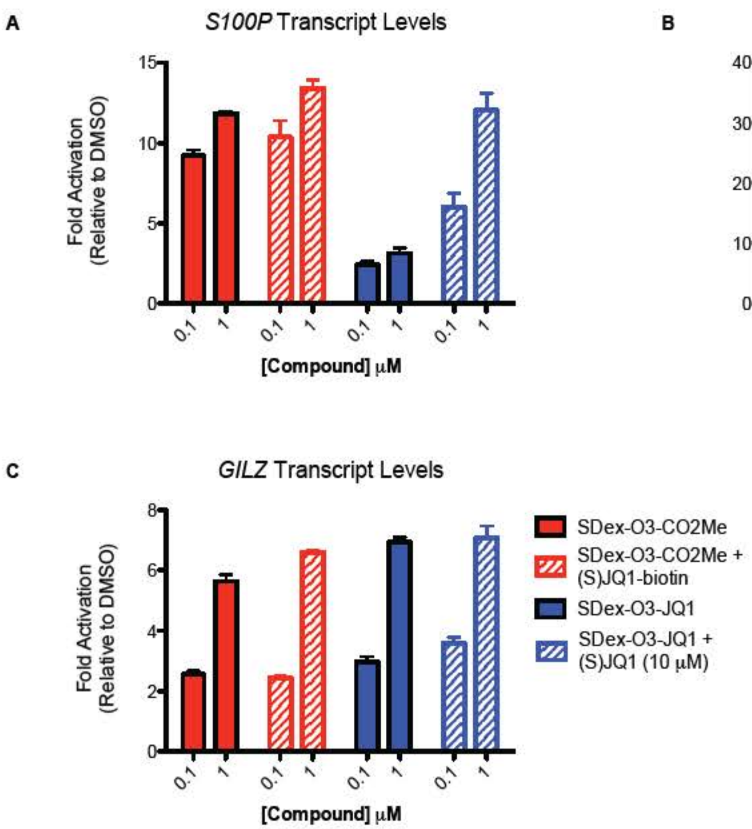


Figure 14 Effects of BRD4 recruitment on endogenous gene transcription. The effects of the designed ligands on the transcription of *S100P* (A), *FKBP5* (B), and *GILZ* (C) in transfected U2OS cells are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method.

The human adenocarcinoma lung epithelial cell line A549 expresses endogenous GR and is commonly used as a model line for the study of GR actions and activity. The effects of SDex-O3-JQ1 treatment on transcriptional activity in A549 cells was investigated to determine if similar patterns are displayed in cells expressing endogenous levels of GR. Comparing the transcription of *S100P* and *GILZ* provided the starkest difference in activity mediated by SDex-O3-JQ1 and provided a template for further studies in A549 cells and, in the future, in PC3 and other prostate cancer systems. Following treatment with the indicated compounds, A549 cells were lysed, total RNA was isolated, and the indicated transcripts were quantified. Transcriptional activity is displayed as fold activation relative to transcript levels in vehicle-treated cells

(Fig. 15). The activation patterns of *S100P* (Fig. 15A) in A549 cells resemble the profile produced in transfected U2OS cells. Treatment with SDex-O3-CO2Me in the absence or presence of (S)-JQ1 produced a strong agonistic response, while treatment with the bifunctional SDex-O3-JQ1 activated transcription to approximately 50% of the level induced by the monofunctional glucocorticoid. As observed before, co-treatment of SDex-O3-JQ1 with an excess of (S)-JQ1 produces transcriptional activity nearly identical to SDex-O3-CO2Me, implying that (S)-JQ1 is capable of competing away BRD4 recruitment and causing SDex-O3-JQ1 to act as its parent, monofunctional ligand. Similarly, the pattern of *GILZ* transcription (Fig. 15B) resembled the activities produced in transfected U2OS cells, wherein each of the described compounds and combinations produced nearly identical responses.

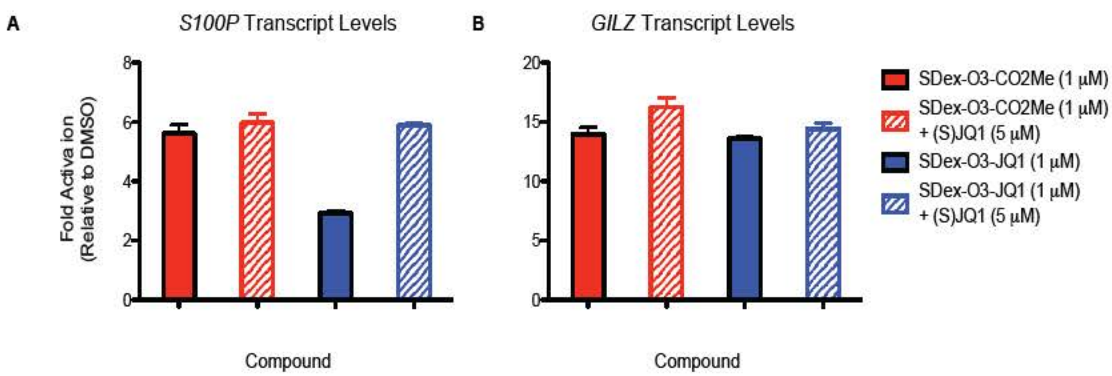


Figure 15 Transcriptional modulation in A549 cells. The effects of the designed ligands on the transcription of *S100P* (A) and *GILZ* (B) in A549 cells are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method.

<u>*Gain of function bifunctional recruiters based upon RU-486.*</u> In the previous funding period (Year 2) we demonstrated that RU486-JQ1 conjugates functioned in reporter contexts. More recently we examined a larger group of genes to assess how broadly those effects extend and if they are indeed dependent upon an

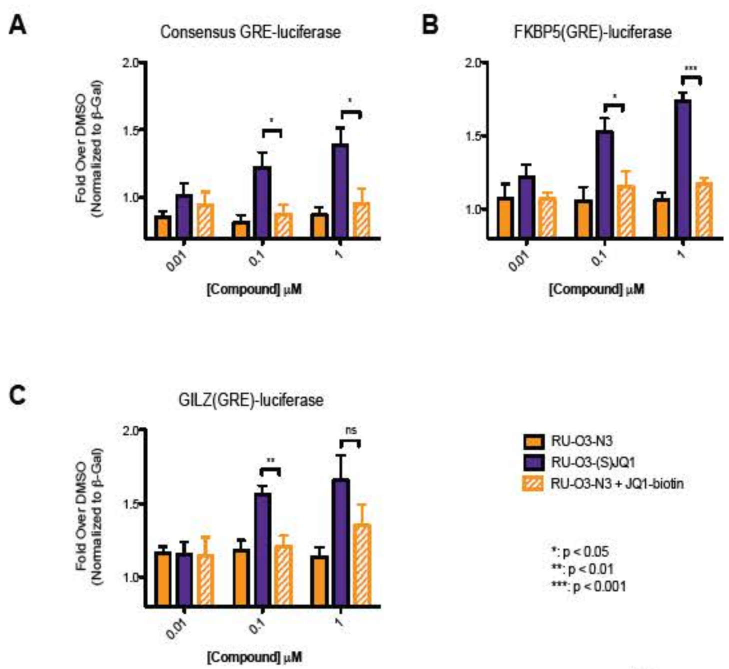


Figure 6. Transcriptional activity of full length human GR in the presence of bifunctional recruiters. Full-length GR was utilized in a transcriptional reporter experiment, with a reporter plasmid using a consensus GRE (A), *FKBP5* GRE (B), or *GILZ* GRE (C).

interaction with Brd4. U2OS cells were transfected with an expression plasmid coding for hGRα and a luciferase reporter plasmid driven by a promoter containing a consensus GRE (Fig. 16A), a GRE from the *FKBP5* sequence (Fig. 16B), or a GRE from the *GILZ* promoter (Fig. 16C). The 'trans' addition employed in this set of trials contained a biotinylated version of (S)-JQ1, acknowledging its utility as a better mimic of the effects of synthetic conjugation on (S)-JQ1. Following treatment, cells were lysed and luciferase levels were quantified and displayed relative to those in vehicle-treated cells. Regardless of the GRE employed, the monofunctional ligand RU-O3-N3 failed to appreciably elevate transcription of luciferase in both the absence and presence of (S)-JQ1-biotin. However, the bifunctional ligand produced a dose-dependent activation of transcription in all three systems, significantly raising transcriptional levels at concentrations as low as 0.1 μM. This was an exciting observation that RNAseq experiments will contextualize and extend.

Assessing context specificity via RNAseq The data described in the previous section in addition to the results from the Year 2 progress report indicate that the activity patterns of the bifunctional molecules are similar in different cell lines and, further, that there is considerable context specificity from gene to gene. In other words, a given bi-functional recruiter may function as an activator, a repressor or have no effect at any given gene and, thus far, we have seen no conceptual principles emerge that enable prediction of these patters. Based upon this and other lines of evidence, we elected to carry out an RNAseq experiment in glucocorticoid-sensitive and resistant matched cell lines (MM1S and MM1R) in order to obtain a complete picture of context specificity; further supporting this choice of cell lines is the expertise of our collaborator Dr. Jay Bradner with this specific model system and the wealth of data for JQ1 and derivatives. Briefly, cells were treated with 1 or 10 μM bifunctional molecules for 4 hours followed by isolation of total mRNA by standard methods. This sequences has been completed and we are in the process of full data analysis, at which point we will publish the bulk of the remaining data. Additionally, we will use these data to define the best path forward for targeting AR in the context of prostate cancer.

Summary of* Task 4 *goals The primary goal of Task 4 was to complete thorough mechanistic investigations of the bifunctional recruiters to demonstrate engagement of both targets. The experiments outlined in the original SOW were centered around HDACi-based recruiters. As the design of the molecules changed to include Brd4-targeting molecules, the specific experiments altered but the goal of demonstrating the bifunctional recruitment mechanism remained.

Summary of* Tast 4 *accomplishments As illustrated below, a variety of biochemical and cellular experiments demonstrate that in the context of agonist-based bifunctional molecules, binding to the nuclear receptor and to Brd4 is accomplished by the bifunctional molecules and, further, that this is important for function. As will be discussed in more detail in the final report, for antagonist-based structures such as RU-486, the attenuated binding affinity that results from even carefully designed conjugation is likely perturbing the system enough that transcriptional effects are minimal. Our data overall suggest that if this can be overcome (and it should be through the synthesis of analogs), the bifunctional recruitment strategy will be readily portable to other nuclear receptors.

Bifunctional molecules interact with receptor and Brd4 in the cellular milieu To first determine if the designed SDex-O3-(S)JQ1 is capable of dimerizing GR and BRD4 in the absence of DNA-binding, ligand-induced coimmunoprecipitation experiments were performed. The human osteocarcinoma U2OS cell line lacks detectable GR expression and was chosen for this study. U2OS cells were transfected with an expression plasmid coding for multiple myc-tagged version of human GR, hGR-myc6, and incubated with the indicated compound. Samples were subsequently lysed and incubated with magnetic beads coated in BRD4-recognizing antibody (αBRD4). Bound proteins were eluted and analyzed by Western blot for the presence of hGR-myc6 using a myc-recognizing antibody (Fig. 17) SDex-O3-(S)JQ1 induced the coimmunoprecipitation of GR with BRD4. Neither the vehicle (DMSO) or linker (SDex-O3-CO2Me)

incubated-samples result in the coimmunoprecipitation of hGR-myc6, precluding the possibility of a non-specific interaction between GR and BRD4. Additionally, the inactive diastereomer, SDex-O3-(R)JQ1, was incapable of coimmunoprecipitating hGR-myc6, strongly supporting the notion that the observed interaction is being specifically modulated through the interaction of each portion of the bifunctional molecule with its target binding pocket.

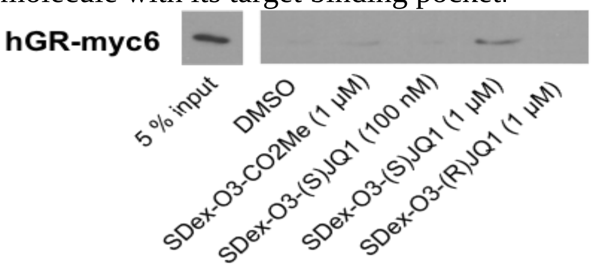


Figure 17 Dimerization of hGR and BRD4. Co-immunoprecipitation experiments were performed to detect the ability of the bifunctional molecule to dimerize hGR and BRD4. Cellular

SDex-JQ1 function is dependent upon GR LBD and Brd4 To investigate the effects of agonist-facilitated BRD4 recruitment on transcription, we employed a traditional three-hybrid experiment as we had used previously with the RU486-based molecules (Year 2 progress report). This arrangement additionally allows us to confirm that SDex-O3-JQ is capable of chemically dimerizing GR and BRD4 in a cellular system. HeLa cells expressing endogenous levels of BRD4 were transfected with a luciferase reporter plasmid bearing five Gal4 DNA-binding sites and an expression plasmid for a Gal4-GR(LBD) chimera and treated with the indicated compounds. The resulting activation of transcription is displayed in Fig. 18B as a fold activation of luciferase produced over the levels in vehicle-controlled cells. As expected, the monofunctional SDex-O3-CO2Me acted as an agonist in this system, likely activating transcription through its induced rearrangement of the GR(LBD) AF2 domain. As was seen with the recruitment of VP16-FKBP, the bifunctional SDex-O3-JQ1 acts as a ‘superactivator’ of transcription, stimulating the expression of luciferase approximately 3-fold higher than the maximum activity displayed by SDex-O3-CO2Me. A squelching experiment was subsequently performed with JQ1 to determine if BRD4 recruitment is the likely mechanism for producing this activity level. HeLa cells were transfected as above and treated with SDex-O3-JQ1 (1 μM) along with increasing levels of either (S)-JQ1 or inactive (R)-JQ1. As seen in Fig. 18C, increasing levels of (S)-JQ1 suppressed the transcriptional activity of SDex-O3-JQ1 in a dose-dependent fashion, with high concentrations of (S)-JQ1 (10 μM) suppressing activity approximately 70%. However, co-treatment with (R)-JQ1 did not produce this effect. This observation supports the hypothesis that BRD4 recruitment is causing the marked difference in activity between SDex-O3-CO2Me and SDex-O3-JQ1; however, (S)-JQ1 is capable of interfering with dexamethasone-induced transcription (unpublished observations).

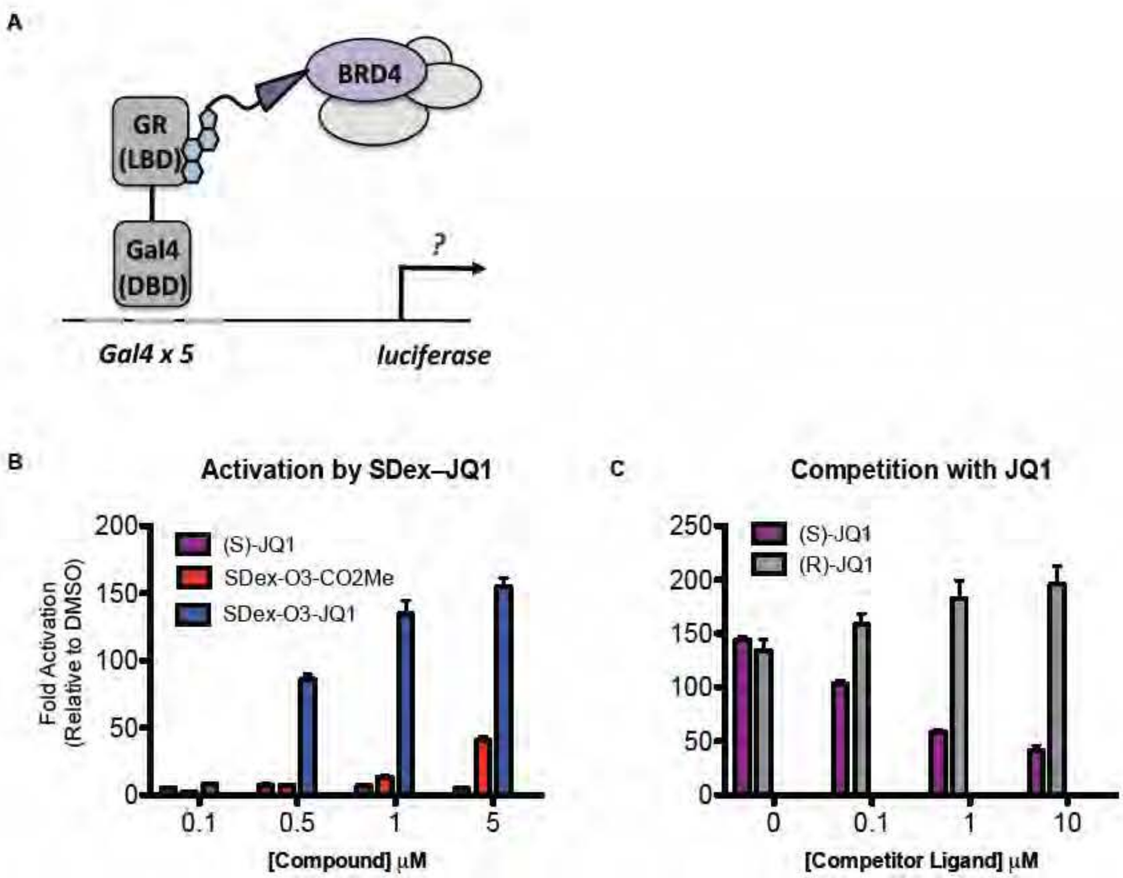


Figure 18 Mammalian three-hybrid targeting an endogenous protein. A three-hybrid experiment, using a Gal4-GR(LBD) chimera, relying on endogenous BRD4 recruitment to drive activity was constructed (A). B: the monofunctional GR ligand acted as a partial agonist, while bifunctional ligand strongly and potently activated transcription. C: the enhanced activity of the bifunctional molecule was dependent on BRD4 recruitment, as determined by squelching experiments.

KEY RESEARCH ACCOMPLISHMENTS

• Developed and validated a flexible, portable synthetic methodology for the preparation of bifunctional molecules. (Appendix 1)

• Two bifunctional molecules that both bind to androgen receptor and function as HDAC inhibitors were developed through the Task 1 workflow. (Appendix 1)

• Identified an agonist-based scaffold that extrinsically regulates full-length, native nuclear receptor at endogenous promoters in more than one cellular context. Importantly, this scaffold shows considerable context specificity.

• Biochemical and cellular experiments (Task 4) support a model in which the agonist-based bifunctional recruiters function through interaction with both binding partners. **This validates the overall strategy that was the goal of this proposal**.

REPORTABLE OUTCOMES

• A manuscript outlining the findings of Task 3 and 4 is currently in preparation: Van Dyke, A.; Carolan, J.P.; Qi, J.; Pawlik, J.; Bradner, J. Mapp, A.K.

• Data from all three tasks have been presented by (former) postdoctoral fellow and current Assistant Professor Dr. Aaron Van Dyke at the 2014 Bioorganic Chemistry Gordon Research Conference. Graduate student JP Carolan has presented this work at the 2014 Life Sciences Institute Poster session (Nov 2014), the 229th American Chemical Society National Meeting in San Francisco, the 2014 Vaughn Symposium at the University of Michigan. Graduate student Steven Sturlis has also presented this work. Appendix 2 contains the abstracts.

CONCLUSIONS

The overall goal of this research project was to develop and implement a conceptually innovative strategy for down-regulating androgen receptor: the creation of bifunctional molecules that simultaneously bind to the androgen receptor and to chromatin modifying complexes. In this way, AR function can be extrinsically controlled. Over the course of the project, we successfully developed this strategy from a model system (Appendix 1) to targeting endogenous nuclear receptor in the native context (manuscript in preparation). We identified that maintaining affinity for the receptor (AR or other receptor) is critical for the success of a given bifunctional molecule scaffold, and, as such, additional molecule optimization is needed to translate this approach to native androgen receptor targeting.

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2014 The Best of Basic Research

Bifunctional Ligands Allow Deliberate Extrinsic Reprogramming of the Glucocorticoid Receptor

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Therapies based on conventional nuclear receptor ligands are extremely powerful, yet their broad and long-term use is often hindered by undesired side effects that are often part of the receptor's biological function. Selective control of nuclear receptors such as the glucocorticoid receptor (GR) using conventional ligands has proven particularly challenging. Because they act solely in an allosteric manner, conventional ligands are constrained to act via cofactors that can intrinsically partner with the receptor. Furthermore, effective means to rationally encode a bias for specific coregulators are generally lacking. Using the (GR) as a framework, we demonstrate here a versatile approach, based on bifunctional ligands, that extends the regulatory repertoire of GR in a deliberate and controlled manner. By linking the macrolide FK506 to a conventional agonist (dexamethasone) or antagonist (RU-486), we demonstrate that it is possible to bridge the intact receptor to either positively or negatively acting coregulatory proteins bearing an FK506 binding protein domain. Using this strategy, we show that extrinsic recruitment of a strong activation function can enhance the efficacy of the full agonist dexamethasone and reverse the antagonist character of RU-486 at an endogenous locus. Notably, the extrinsic recruitment of histone deacetylase-1 reduces the ability of GR to activate transcription from a canonical GR response element while preserving ligand-mediated repression of nuclear factor-κB. By providing novel ways for the receptor to engage specific coregulators, this unique ligand design approach has the potential to yield both novel tools for GR study and more selective therapeutics. (*Molecular Endocrinology* 28: 249–259, 2014)

Synthetic glucocorticoids are one of the most widely used pharmacological agents, mainly because of their potent antiinflammatory and immunosuppressive effects. Given that maladaptive inflammation or inappropriate immune responses are a central part of many chronic diseases, glucocorticoids are an invaluable therapeutic tool in a wide range of conditions including arthritis, asthma, lupus, and allergy and are an important element of immunosuppressive regimens for organ transplantation (1). Despite these well-established and sometimes life-saving therapeutic applications, conventional glucocorticoid therapy is severely limited due to undesirable side effects. These are mainly due to the profound metabolic changes in energy and protein metabolism that endogenous glucocorticoids set in motion as an adaptive response to transient stress. Consequently, pharmacological glucocorticoid excess leads to hyperglycemia, visceral adiposity, and insulin resistance as well as muscle wasting and osteoporosis. Pharmacological approaches that mitigate the metabolic effects of glucocorticoids while preserving their immunomodulatory activity would be a major therapeutic advance. This, however, has proven elusive despite intense efforts (2–4).

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Abbreviations: AF-2, activation function-2; Dex, dexamethasone; HDAC, histone deacetylase; HEK, human embryonic kidney; FKBP, FK506 binding protein; GR, glucocorticoid receptor; LBD; ligand binding domain; NF-κB, nuclear factor-κB; NLS, nuclear localization signal; SDex, derivative of Dex that allows facile conjugation with other molecules; VP16, virion protein 16.

The effects of glucocorticoids are mediated by the glucocorticoid receptor (GR), a prototypic member of the nuclear receptor superfamily of sequence-specific, ligand-regulated transcription factors. Upon binding of an agonist to the C-terminal ligand binding domain (LBD), the GR translocates to the nucleus and localizes to specific loci through a central zinc finger region capable of direct recognition of specific sequences or through tethering to other transcription factors. From these sites, the GR influences the transcription of target genes by nucleating the assembly of specific coregulatory complexes through protein-protein interactions (5). The receptor orchestrates this process by integrating multiple signals (6), including variations in the target DNA sequence (7), intracellular signaling cascades, posttranslational modifications (8, 9), and uniquely, small cell-permeable ligands that bind to the LBD (Figure 1A).

The canonical mode of action of endogenous ligands involves their binding to the LBD and consequent reorientation of helix 12, leading to the engagement of the C-terminal activation domain [activation function-2 (AF-2)]. In concert with additional activation functions in the N-terminal region, these conformational changes alter the interaction surfaces for transcriptional coactivators and corepressors that are responsible for controlling chromatin remodeling as well as transcriptional initiation and elongation (10).

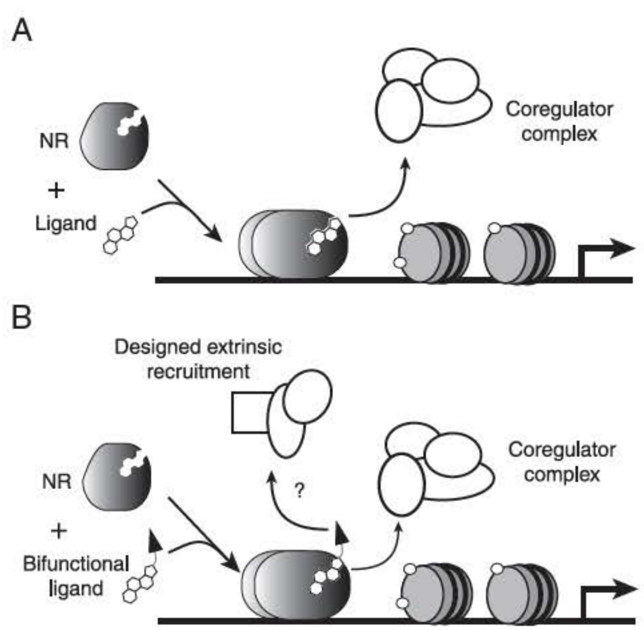

Figure 1. Extrinsic control of nuclear receptors. A, Transcriptional regulation by conventional ligands involves binding to the nuclear receptor (NR) and subsequent nucleation of coregulator complexes. The spectrum of targeted complexes is dictated mainly by the intrinsic conformation of the LBD induced by the ligand. B, Bifunctional ligands with unique targeting functionalities may allow the selective recruitment of coregulator complexes not accessible to conventional ligands.

Glucocorticoid responses are the integration of complex patterns of tissue-specific gene expression and involve both activation and repression of target genes (11, 12). Limiting metabolic side effects while preserving immunomodulatory actions therefore would require a clear identification of the on-pathway desirable responses as well as those involved in undesirable side effects and, importantly, an effective means to elicit one without the other. The ability of glucocorticoids to repress expression of multiple proinflammatory cytokines is a central component of its antiinflammatory effects, whereas the induction of metabolic enzymes such as phosphoenolpyruvate carboxykinase is an important component of the metabolic response. Initial efforts have therefore focused toward dissociated agonists that can support repression while minimizing transactivation but have met with limited success (2–4).

The difficulties associated with conventional nuclear receptor ligands are likely to be due in large part to the fact that they act solely through the allosteric modulation of receptor conformation and are therefore constrained to recruit from a closed set of cofactors that are within the intrinsic interaction envelope of the receptor. This puts a limit to the range of achievable functional effects. Furthermore, despite the incorporation of novel protein dynamics criteria (13), it is not yet possible to rationally design into a conventional ligand the ability to recruit specific coregulator complexes or to generate a particular pattern of gene expression. A further challenge is that coactivators involved in transactivation such as GR-interacting protein-1 also participate in transcriptional repression mechanisms (14, 15). Furthermore, the development of clinical resistance is a significant problem, particularly in cancer therapy (16).

In a significant departure, we describe here a novel strategy that circumvents the limitations of conventional allosteric ligands and frees the receptor to engage in novel extrinsic functional interactions that are open to rational design. The approach leverages the versatility of GR bifunctional ligands, which, although have proven useful in three hybrid assays (17), have never been exploited to regulate the intact GR in its native context. By linking prototypic agonist and antagonist GR ligands to the small molecule FK506, we have thus generated bifunctional ligands that can bind the intact receptor and bridge it to designed transcriptional activators or corepressors containing the FK506 binding protein (FKBP) domain (Figure 1B). Using this strategy, we demonstrate for the first time that the regulatory repertoire of the native receptor at endogenous genomic loci can be expanded by directing the deliberate recruitment of extrinsic coregulators. This strategy allows the designed reprogram-

ming of the intact receptor and can dramatically enhance or completely reverse the efficacy of conventional ligands. By eliciting unique responses, this new class of GR ligands can thus serve as mechanistic probes to discern which are the relevant on-pathway transcriptional responses required for specific therapeutic effects and which are not required or causative of undesired side effects (18). Notably, this work demonstrates the viability of exploiting druggable small molecule binding sites in coregulator complexes for extrinsic recruitment to the receptor and opens the way for the purposeful recruitment of endogenous transcriptional coactivators and repressors to identify and drive therapeutically desirable responses.

Materials and Methods

Synthesis and binding affinity of GR bifunctional ligands

The macrolide FK506 was installed via a polyethylene glycol linker to a dexamethasone (Dex) derivative in which the 21-hydroxyl group is replaced by a thioether linkage (19). For the RU486 derivatives, a similar linker strategy was used using the aniline group of RU486 as the attachment point. Parallel compounds lacking the FK506 moiety were prepared as controls. Detailed information on compound synthesis is included as Supplemental Data, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. Binding affinities for Dex, RU486, and their derivatives were determined experimentally by radiolabeled competition binding assays using an extract from Hi5 insect cells expressing an N-terminally His- and green fluorescent protein-tagged rat GR. For extract generation, Hi5 cells were infected with H10 eGFP TEV GR baculovirus at a multiplicity of infection of 4 and lysed 48 hours after infection with a Dounce homogenizer in 20 mM HEPES (pH 7.4), 1 mM EDTA, 5% glycerol, 20 mM sodium molybdate, 5 mM dithiothreitol, and Complete (Roche) protease inhibitor cocktail (one tablet per 10 mL). The homogenate was centrifuged at 60 000 rpm at 4°C for 30 minutes in a TI-35 rotor (Beckman). The clear supernatant was aliquoted, flash frozen, and stored at −80°C until use. Binding assays were carried out in 10 mM HEPES (pH 7.4), 1 mM EDTA, and 20 mM sodium molybdate buffer in a 96-well plate format. The receptor extract (30 μg/well) was incubated with a mixture consisting of 10 nM 1,2,4,6,7-[^{3}H]dexamethasone (PerkinElmer) and increasing concentrations of test ligand at 4°C in a final volume of 50 μL. After a 2-hours incubation, 100 μL of a dextran coated charcoal solution (1% charcoal; 0.2% dextran in 10 mM HEPES, pH 7.4; 1 mM EDTA) was added to each well, followed by centrifugation for 2 minutes at 1000 $\times$ g. Aliquots (80 μL) of the resulting supernatants were transferred to Optiplate 96-well plates (PerkinElmer), supplemented with 120 μL of MicroScint 40 (PerkinElmer) and read on a TopCount NXT microplate scintillation counter. Data were fit to a single binding site competition model using GraphPad Prism version 5.0 (GraphPad Software).

Plasmids, cell culture, and transfections

Plasmids p6R GR (20) and pRSV β-gal (21) are Rous sarcoma virus promoter-driven expression vectors for wild-type rat GR and β-galactosidase, respectively. pGBR 6.1 is a luciferase-based reporter harboring a 500-bp intronic region of the human *FKBP5* gene and has been described previously (11, 22). The 5$\times$ nuclear factor-κB (NFκB) luciferase reporter (23) was a kind gift of Dr Gabriel Nuñez (University of Michigan). Plasmids pLIC FKBP and pNLS-FKBP are cytomegalovirus promoter-driven expression vectors for a protein bearing the Simian virus-40 nuclear localization signal (NLS) followed by human FKBP1A. The VP16 activation domain sequence (residues 411–456) was inserted between the NLS and FKBP1A sequences of pNLS-FKBP to generate pNLS-VP16-FKBP. A similar strategy was used to insert the mouse histone deacetylase (HDAC)-1 sequence at the same position of pLIC FKBP. For all constructed plasmids, relevant regions were sequenced and are available upon request. Human embryonic kidney (HEK) 293T cells were cultured in DMEM containing 10% fetal bovine serum (Life Technologies).

For functional assays, 3×10^6 cells seeded in 100-mm plates were transfected 24 hours later with 50 ng of p6R GR, 400 ng pGBR 6.1, 200 ng pCMV β-galactosidase, and 200 ng of pNLS-FKBP, pNLS-VP16-FKBP, or pLIC HDAC1-FKBP using Lipofectamine (Life Technologies) according to the manufacturer's instructions. Cells were trypsinized 16 hours after transfection and resuspended in DMEM supplemented with 10% heat-inactivated, charcoal-stripped fetal bovine serum and seeded onto 96-well plates at a density of 2×10^4 cells/well. After an additional 8 hours, cells were treated with either vehicle or the indicated ligands and harvested 16 hours later. Cells transfected with the 5$\times$ NF-κB luciferase reporter plasmid were treated with 10 ng/mL of human TNFα (Sigma) in addition to the indicated ligands. Luciferase and β-galactosidase activities were determined as described previously (20). For endogenous gene expression analysis, cells (1.5×10^4/well) were seeded onto 24-well plates and transfected 16 hours later with 50 ng of p6RGR and 25 ng of pLIC FKBP, pNLS VP16 FKBP, or pLIC HDAC1-FKBP using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Sixteen hours after transfection, cells were treated for an additional 6 hours with either vehicle or the indicated compounds. Total RNA was isolated using RNeasy RNA isolation kits (QIAGEN), and 500 ng of each RNA sample was used to synthesize cDNA using iScript cDNA synthesis kits (Bio-Rad Laboratories).

Quantitative real-time PCRs were carried out in duplicate in a Roche 480 LightCycler using QuantiTect SybrGreen reagents (QIAGEN) and primers for human RPL19 (forward, 5′-ATGTATCACAGCCTGTACCTG-3′; reverse 5′-TTCTTGGTCT CTTCCTCCTTG-3′) and S100P (forward, 5′-CGGAACTAGAGACAGCCATGGGCAT-3′; reverse 5′-AGACGTGATT GCAGCCACGAACAC-3′) genes. LinRegPCR (version 11.0) (24) software was used to estimate S100P mRNA levels relative to the reference RPL19 transcript. For protein level analysis, parallel cultures were harvested directly in SDS-PAGE sample buffer. After brief sonication, lysates were centrifuged for 2 minutes at 16 000 $\times$ g, and supernatants were resolved by SDS-PAGE and transferred to polyvinyl difluoride membranes (Millipore). FKBP12 (Abcam ab58072) and BuGR2 antibodies were used at 1:2000 dilution. Images were captured in a Li-Cor Odyssey Fc reader using stabilized goat antimouse horseradish per-

oxidase-conjugated antibodies (Pierce) and Super Signal West Femto chemiluminescence reagents (Pierce).

Results

Design of bifunctional ligands

Using the GR as a paradigm and building on the significant experience in structure-activity relationship for GR ligand conjugates (17, 19, 25–28), we created bifunctional molecules based on the agonist Dex and the antagonist RU486. The least perturbing derivative of Dex that allows facile conjugation with other molecules has been termed SDex, in which the 21-hydroxyl group is replaced by thioether linkages (19) (Figure 2). RU486 has been linked to bile acids through its aniline group and retained antagonistic activity (28). Using a polyethylene-glycol linker as a spacer, we have thus conjugated these ligand derivatives to the natural product FK506 (Figure 2). Our choice was based on the extensive experience using FK506 conjugates for ligand-induced protein complex assembly (29, 30). Compounds bearing a linker only were synthesized as controls (Figure 2).

The binding affinity of the conjugates for full-length GR was determined in a radiolabeled Dex competition binding assay. As can be seen in Figure 3A, the affinities of SDex-O_3-OMe and SDex-O_2-FK506 are approximately 50 and 100 nM, respectively. Although this is 10- and 20-fold lower than the affinity of Dex (5 nM), these values are in a range comparable with the affinity of endogenous steroids such as cortisol (20 nM). Although the conjugation of FK506 to SDex lowers the binding affinity a modest 2-fold, this difference is not due to effects of binding cellular FK506-binding proteins because binding studies done in the presence of free FK506 yielded similar results (data not shown). In the case of the RU486 derivatives, both compounds displayed comparable affinities (~90 nM), which are less than an order of magnitude (~6-fold) lower than that of unmodified RU486 (Figure 3B). From this analysis, it is clear that the synthetic strategy yielded bifunctional ligands that retain a significantly high affinity for GR.

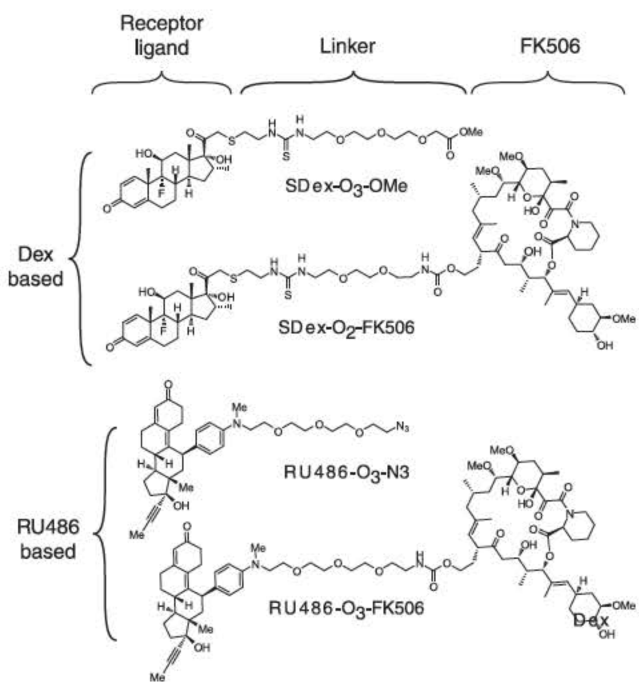

Figure 2. GR bifunctional ligands. Structures of GR bifunctional ligands based on the agonist dexamethasone (top panel) or the antagonist RU486 (bottom panel). Receptor binding, linker, and FK506 moieties are indicated.

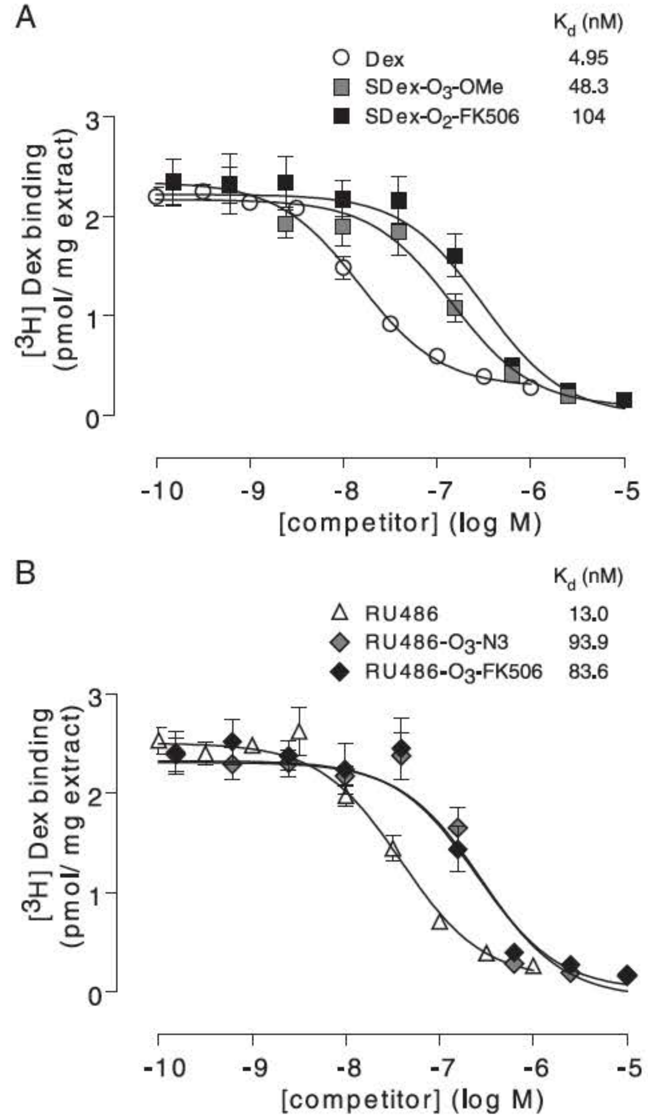

Figure 3. Bifunctional ligands bind to the GR. Competition binding assays based on displacement of ^{3}lsqb]H]Dex (10 nM) from GR were carried out as described in *Materials and Methods*. All curves are fits to a competitive single binding site model. Values for the calculated dissociation constant (K_d) are indicated in the inset. A, Competition using Dex- or SDex-derived ligands. B, Data for RU486-based ligands.

Extrinsic recruitment of a designed coactivator enhances GR ligand efficacy

FK506-binding proteins such as human FKBP1A bind with subnanomolar affinity to FK506, and the fact that this high-affinity interaction is retained, even when the macrolide is conjugated to other molecules, has made this pairing the basis for multiple successful small molecule-mediated protein recruitment strategies (31). As a first approach, we designed and constructed a coactivator fusion protein consisting of a nuclear localization signal, the strong transcriptional activation domain of the herpes simplex virion protein 16 (VP16), and FKBP1A and examined its ability to modulate GR activity by monitoring the transcriptional output of a GR-stimulated reporter driven by a natural GR enhancer sequence. As can be seen in Figure 4A, in the absence of any fusion protein, the Dex-derived bifunctional molecules (SDex-O_2-FK506 and SDex-O_3-OMe) led to a dose-dependent enhancement of activity with both compounds achieving maximal responses comparable with that of the parent agonist Dex. As expected from their somewhat reduced binding affinity, these compounds activated with lower potency relative to Dex. Interestingly, even though SDex-O_2-FK506 has discernibly lower affinity than SDex-O_3-OMe, both compounds activated with comparable potencies, suggesting an advantage for the FK506 conjugate. From these data, it is apparent that despite the presence of the linker and FK506 moiety, the bifunctional ligands remain cell permeable and retain full efficacy, revealing a significant degree of steric tolerance by the native receptor.

In notable contrast, in the presence of the VP16-FKBP fusion (Figure 4B), the FK506-conjugated ligand (SDex-O_2-FK506) displayed a nearly 2-fold increase in maximal activity relative to Dex, and the corresponding EC_{50} of 0.76 nM reflects an approximately 7-fold increase in potency relative to the linker-only ligand (SDex-O_3-OMe). Notably, these effects were specific to the FK506-conjugated ligand because the presence of the fusion did not appreciably alter the response to Dex or SDex-O_3-OMe. Thus, the enhancement depends on both the fusion and the FK506 moiety of the ligand. These data argue that the enhanced activation in the presence of the fusion is due to extrinsic recruitment by the bifunctional ligand. If this is indeed the case, it can be anticipated that the effect should be disrupted by excess unconjugated FK506 (32). In support of this prediction, increasing concentrations of free FK506 reduced the activity of the bifunctional ligand (Figure 4C) until it was essentially indistinguishable from the behavior of the ligand lacking FK506 (except for a small increase in basal activity at the highest concentration of FK506). As expected, the dampening effect of free FK506 depended on the presence of the fusion and was not observed for SDex-O_3-OMe (data not shown). Taken together, these results clearly indicate that by using the extrinsic recruitment strategy, bifunctional ligands endow GR with the ability to activate transcription well beyond what can be achieved with one of the most efficacious conventional ligands. This strategy opens the way for uniquely high-efficacy ligands that could serve to overcome clinical resistance or to restore function due to inborn deficits in GR transactivation.

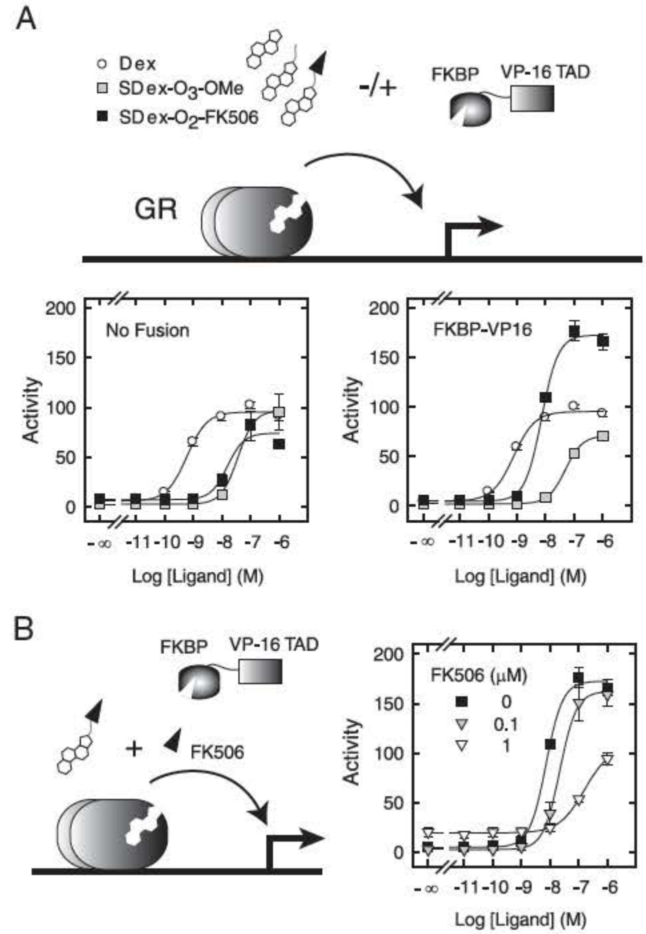

Figure 4. Directed enhancement of efficacy using bifunctional GR ligands. Dose-response curves for transcriptional activation by Dex, SDex-O_3-Ome, and SDex-O_2-FK506 in HEK 293T cells expressing GR alone (A), or coexpressing GR and the fusion protein FKBP-VP16 (B). Note the bifunctional ligand and fusion-dependent enhancement in efficacy. C, Dose-response curves for SDex-O_2-FK506 in the absence (filled squares) or presence of 0.1 (gray triangles) or 1 μM (open triangles) of free FK506 in cells coexpressing GR and the fusion protein FKBP-VP16. Note that at the highest concentration of FK506, the response reverts to that seen in the absence of extrinsic recruitment. Data represent the average ± SEM of at least three experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex.

Extrinsic recruitment of HDAC1 selectively reduces agonist efficacy in activation but not repression contexts

Therapeutically, GR ligands that have reduced transactivation efficacy but retain full agonism in repression

contexts have been sought after because they may display more favorable efficacy vs side effect profiles. Because these properties have proven to be difficult to obtain using conventional ligands, we sought to build on our initial results and use the extrinsic control approach to explicitly design and implement this desired regulatory outcome. Because recruitment of corepressor complexes is a common strategy used by transcription factors to negatively regulate transcription, we constructed a designed coregulator in which FKBP1A is fused to the histone deacetylase HDAC1, an enzyme that is an integral component of multiple corepressor complexes (33, 34). In the presence of this coregulator, we then examined the ability of SDex-based bifunctional ligands to mediate GR activity in both a canonical activation context (as examined in the experiments above, Figure 5A, left panel) as well as in a repression context (Figure 5A, right panel) in which GR inhibits TNFα-stimulated NF-κB activity. As in the case of proinflammatory cytokine genes, GR is recruited to DNA indirectly by tethering to NF-κB and inhibits transcription in an agonist-dependent manner, likely at a step downstream of RNA polymerase II recruitment (35, 36). As can be seen in Figure 5B, left panel, in the presence of the FKBP-HDAC1 coregulator, the maximal response elicited at the activation context by SDex-O_2-FK506 was significantly blunted, reaching only approximately 30% of the response induced by Dex. In contrast, the fusion did not alter the activity elicited by either Dex itself or SDex-O_3-OMe (compare with Figure 4). These results indicate that the extrinsic recruitment of HDAC1 can successfully oppose the intrinsic efficacy of a conventional ligand in an activation context. In notable contrast, analysis of the tethering repression context (Figure 5B, right panel) revealed that both the control and FK506-conjugated SDex bifunctional ligands supported GR-mediated inhibition of NF-κB to the same extent as Dex (Figure 5B). Because this same repression activity profile was observed in the absence of any fusion protein (data not shown), this indicates that the FKBP-HDAC1 coregulator did not interfere with the ability of the ligands to repress in this context. As expected, given their lower intrinsic affinity, the bifunctional ligands were less potent than Dex in both the activation and repression contexts. Taken together, the results indicate the successful establishment of the desired outcome and underscore the deliberate design potential of the extrinsic recruitment approach.

Efficacy switch from antagonist to agonist through extrinsic recruitment

The above experiments show that in an activation context, it is possible to positively or negatively modulate the efficacy of an agonist ligand by the judicious extrinsic

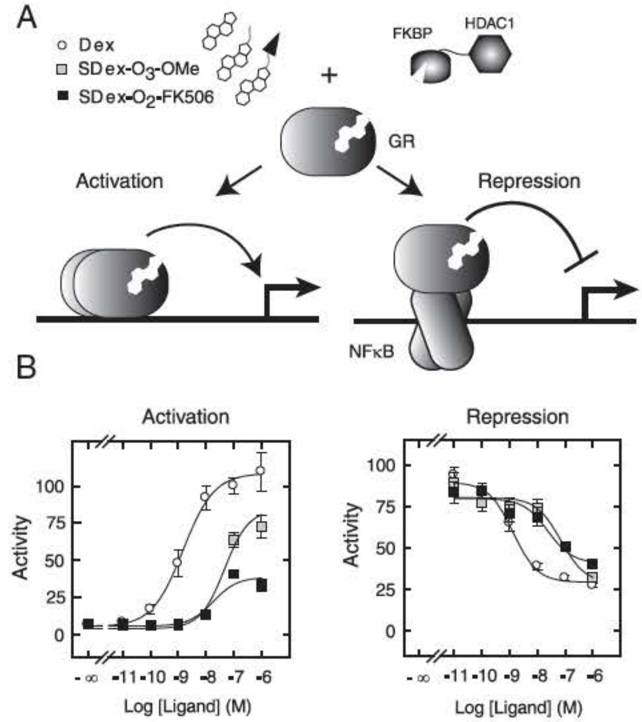

Figure 5. Suppressed transactivation with preserved transrepression using extrinsic recruitment. A, Diagram depicting the GR-mediated activation (left panel) and repression (right panel) contexts for functional assays. The bifunctional ligands with associated legends are also shown. B, Dose-response curves for transcriptional activation by Dex, SDex-O_3-Ome, and SDex-O_2-FK506 in HEK 293T cells coexpressing GR and the fusion protein HDAC1-FKBP are shown on the left (see Figure 4 for comparison). Transcriptional repression of TNFα stimulated NF-κB activity by Dex and the bifunctional ligands in the presence of the fusion protein HDAC1-FKBP is shown on the right. Data represent the average $\pm$ SEM of at least three experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex (left panel) or 10 ng/mL TNFα alone (right panel). The basal activity in the absence of TNFα was less than 1%.

recruitment of coregulators. To probe the design versatility and scope of this approach, we sought to determine to what extent the regulatory effects of extrinsically recruited factors can be dissociated from the efficacy intrinsic to the GR binding moiety of the bifunctional ligand. To this end, we examined the properties of RU486-based ligands in which the receptor binding moiety is an antagonist. As can be seen in the left panels of Figure 6, in the absence of any fusion, RU486 and its derivatives showed no detectable activation of the GR-responsive promoter (Figure 6A, left panel). The compounds, however, are active and cell permeable because they are able to antagonize the activity of 3 nM Dex in a dose-dependent manner (Figure 6B, left panel). Interestingly, even though both RU486-based ligands have indistinguishable affinities (Figure 3B), the FK506 conjugate antagonized Dex with an IC_{50} approximately 6-fold lower than the linker-only ligand. This makes the FK506 conjugate comparable with

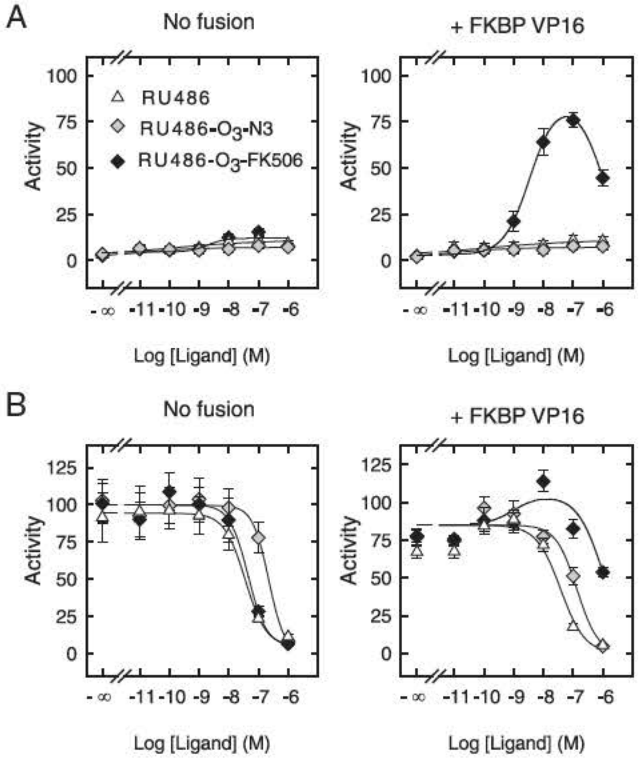

Figure 6. Antagonist to agonist conversion through extrinsic recruitment. A, Dose-response curves for transcriptional activation by RU486-based ligands in HEK 293T cells expressing GR alone (left panel) or coexpressing GR and the fusion protein FKBP-VP16 (right panel). B, Effect of the ligands in the presence of 3 nM Dex. Data represent the average ± SEM of at least three experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex.

the parental RU486, despite its lower binding affinity. This indicates that the FK506 moiety imparts additional properties to the ligand, which could include effects on the cellular accumulation of the ligand.

In contrast to the above data, in the presence of the VP16-FKBP fusion, RU486-O_3-FK506 became an effective inducer of transcriptional activation, reaching a maximal activity nearly as strong as that of Dex (Figure 6A, right panel). Interestingly, the response is biphasic because activation is reduced at the highest concentration. This behavior, however, is consistent with the properties of bifunctional ligands because at sufficiently high concentrations, the formation of binary ligand-protein complexes is favored over the ternary complex (32, 37). It is also notable that the half-maximal activation by the FK506 conjugate occurred at concentrations (EC_{50} ~3 nM) significantly lower than the intrinsic receptor affinity (~90 nM). Importantly, and similar to the case of the Dex derivatives, the activity depends on both the fusion protein and the FK506 moiety and can be disrupted with free FK506 (Figure 6A and data not shown).

Consistent with a large gain in efficacy, the behavior of the ligands in the presence of 3 nM Dex (Figure 6B, right panel) indicated that RU486-O_3-FK506 behaved as an agonist and increased activity beyond 3 nM Dex. The fact that the peak activity is higher than that observed with RU486-O_3-FK506 alone also suggests that at these concentrations, in which mixed occupancy is likely, cooperation between intrinsic (Dex bound GR) and extrinsic (RU486-O_3-FK506 bound GR) mechanisms is occurring. As anticipated from competitive displacement, the behavior at the highest concentrations is comparable with that of RU486-O_3-FK506 alone. Furthermore, the presence of the VP16-FKBP fusion did not appreciably alter the antagonistic behavior of the parental RU486 or the conjugate lacking FK506, indicating that the observed effects require both the appropriate coregulator and the FK506 moiety in the bifunctional ligand. Taken together, these data clearly show that the regulatory effects elicited through extrinsic recruitment can be fully dissociated from the intrinsic properties of the GR binding moiety. The versatility of the approach is such that it allows for the predictable reprogramming of the transcriptional output of GR such that the behavior of a ligand can be completely reversed from an antagonist to an agonist.

Regulation in an intact chromatin environment through extrinsic recruitment

The implementation of the overall GR transcriptional program in vivo occurs in the context of a complex chromatin environment, and any successful ligand strategy must be able to operate under these circumstances. To demonstrate that GR-extrinsic transcriptional control can also be achieved at endogenous GR target genes in their native chromatin context, we focused on the *S100P* gene. The basal expression of this gene in HEK 293T cells is comparatively low and can be stimulated approximately 50- to 100-fold by Dex only upon GR expression. These properties indicate that the receptor is a major determinant of S100P transcription and make it a suitable target for analysis. In cells coexpressing FKBP alone, both of the SDex derivatives at 100 nM (which is comparable with their dissociation constant) were able to activate the *S100P* gene approximately 20-fold (Figure 7A, left panel). In contrast, in cells coexpressing the VP16-FKBP fusion, S100P expression was 3-fold higher in the presence of SDex-O_2-FK506 compared with the control ligand SDex-O_3-OMe, which lacks the FK506 moiety (Figure 7A, center panel). In fact, the activity elicited by the FK506 conjugate reached levels comparable with those obtained with the same concentration of Dex (which in comparison, corresponds to a 20 fold excess over its own dissociation constant). On the other hand, in cells ex-

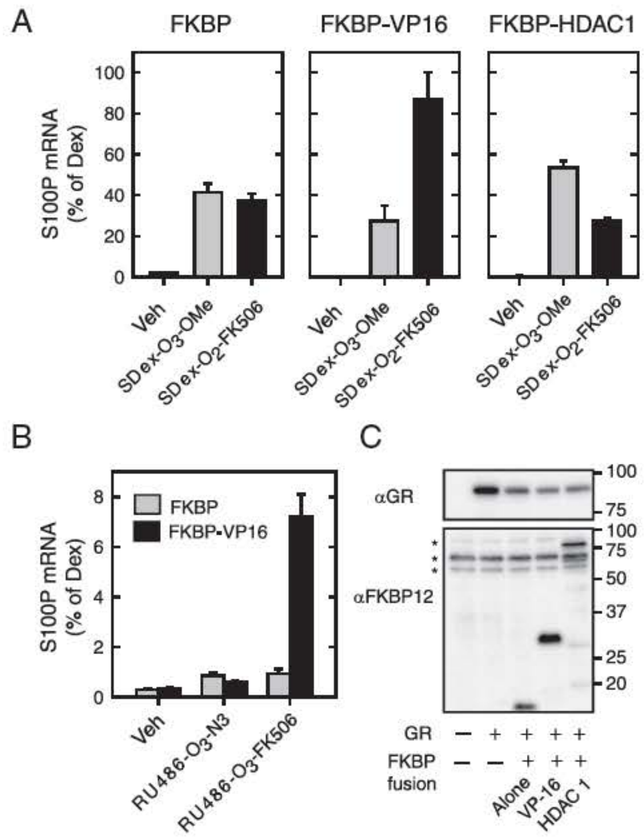

Figure 7. Directed regulation of the endogenous *S100P* gene by bifunctional ligands. A, mRNA levels of the *S100P* gene in response to SDex-O_3-OMe and SDex-O_2-FK506 in HEK 293T cells coexpressing GR and FKBP alone (left panel),VP16-FKBP (center panel), or HDAC1-FKBP (right panel). B, Response to RU486-based ligands in cells coexpressing GR and either FKBP alone (gray bars) or the VP16-FKBP fusion (black bars). Data are the averages ± SEM of at least four independent experiments performed in duplicate and are expressed as a percentage of the levels observed in response to 100 nM Dex. Except for the control FKBP-alone data, all comparisons between linker only and FK506 conjugates were statistically significant (two tailed Student's *t* test, $P \leq .01$). C, Western blot analysis of parallel cultures using anti GR (top panel) or FKBP12 (lower panel). Molecular masses of standards (in kilodaltons) are indicated on the right of each panel, and nonspecific species are indicated by asterisks. Predicted molecular masses for the FKBP, VP16-FKBP, and HDAC1-FKBP fusion constructs are 15.8, 20.3, and 71.1 kDa, respectively. The anomalous migration conferred by the acidic VP16 activation domain has been well described (58).

pressing the HDAC1-FKBP fusion, S100P expression in response to SDex-O_2-FK506 was reduced by half in comparison with SDex-O_3-OMe (Figure 7A, right panel). Thus, for both the positive and negative modulation, successful extrinsic control can be demonstrated in a manner that depends on both the appropriate coregulator partner and the FK506 moiety in the ligand.

A parallel analysis of the effects of the RU486-based ligands (Figure 7B) revealed that in the presence of FKBP alone, the ligands did not appreciably activate S100P expression. This is expected from the intrinsic antagonist nature of RU486. In the presence of the VP16-FKBP fusion, however, the FK506 conjugate successfully activated S100P expression more than 10-fold compared with the ligand lacking the FK506 moiety. Importantly, Western blot analysis demonstrated successful expression of the fusion proteins (Figure 7C, bottom panel) and comparable expression of the receptor (Figure 7C, top panel). Taken together, the data clearly indicate that the extrinsic recruitment approach can be readily used to reprogram the expression of genes in their natural context and thus is amenable to further development for potential therapeutic applications.

Discussion

The ligand design we have implemented demonstrates that it is possible to independently manipulate intrinsic as well as extrinsic pathways of GR control and that their combinatorial coupling via bifunctional ligands can yield a variety of regulatory outcomes in both activation and repression contexts (Table 1). The approach is instructive in multiple ways because it reveals mechanistic features of GR function and opens up numerous design opportunities for its directed manipulation. The ability of the intact GR LBD to accommodate both agonist- and antagonist-based bifunctional ligands is notable because the predicted exit trajectory of the linker from the LBD based on structural information is quite different. For the Dex derivatives, the linker attachment site is very close to the surface and projects outward between helix 3 and 11 on the opposite side of helix 12. The linker is likely accommodated with minor movements of nearby residues such as T739 and Ile 747. The ability of the bifunctional ligands on their own to mount maximal responses comparable with Dex argues that the linker is accommodated while preserving a functional AF-2. For the RU406 derivatives, the linker extends from the aniline moiety, which is responsible for preventing helix 12 from adopting an active conformation and is already solvent exposed. Despite opposite exit points from the LBD and a relatively short linker, both bifunctional ligands are proficient for the recruitment of designed coregulators, which indicates a significant degree of flexibility and steric tolerance. These properties are favorable to the further development of the extrinsic recruitment approach.

The ability of the HDAC1-FKBP coregulator to limit transactivation by GR is also revealing. On the one hand, the inhibitory effect does not appear to be due to steric hindrance because FKBP alone or fusions to other proteins of comparable size are inactive (data not shown). Furthermore, although our data are consistent with the established role of HDAC1 as a component of corepres-

Table 1. Outcomes of Combinatorial Intrinsic and Extrinsic Pathways

Pathway		Maximal Effect (Relative to Dex)	
Intrinsic (Ligand character)	**Extrinsic (Cofactor recruitment)**	**Activation**	**Repression**
Agonist (Dex)	None	100%	100%
	VP16	200%	n.t.
	HDAC-1	30%	100%
Antagonist (RU-486)	None	0%	n.t.
	VP16	75%	n.t.

Abbreviation: n.t., not tested.

sor complexes, recent data based on the functional effects of HDAC1 knockdown have been interpreted as HDAC1 playing a positive role in GR transactivation (38). Whether this reflects indirect effects of HDAC1, or more complex interactions as has been suggested recently (39), remains to be determined. It is also important to note that HDACs can play key scaffolding roles in corepressor complexes that do not depend on their HDAC activity (34, 40). Consistent with this view, initial data indicate that the catalytic activity of HDAC1 is not required for its ability to suppress GR transactivation in the extrinsic recruitment approach (data not shown). This would indicate that the catalytic site of HDAC1 could be targeted by bifunctional ligands to recruit functional corepressor complexes. Importantly, targeting HDAC1 allowed selective reduction of GR transactivation while preserving agonist-mediated repression. Such an outcome is difficult to obtain with conventional ligands because the same AF-2-directed coregulators, such as GR interacting protein-1, can participate in both contexts (15).

The ligand design we have implemented has unique properties that make it amenable to directed design strategies. The nearly independent manipulation of the properties of both the receptor binding moiety and the additional chemical functionality is a significant advantage. This modularity extends the receptor's own design in which the DNA and ligand specificity have divergently evolved to generate distinct receptors with unique properties and functions. The substantial level of flexibility afforded by this approach greatly increases the types of ligands that can be envisioned. Although we have demonstrated a controlled change in efficacy with prototypic agonist and antagonist receptor ligands, the strategy could be combined with compounds with some conventional dissociated properties (2) or the arylpyrazole nonsteroidal series (3) that display some cell- and gene-selective properties to leverage both intrinsic and extrinsic effects. The experiments described here demonstrate that the added functionality afforded by the bifunctional ligand can be used in explicit design efforts to direct a desired transcriptional output and override the intrinsic properties of the receptor binding moiety. This also means that ligands with very high affinity but weak efficacy could be used as scaffolds. Such a strategy could counteract the mild penalty in affinity incurred by the introduction of the linker.

It is also important to note that in addition to affecting the pharmacodynamic properties of a receptor ligand, the linker as well as the additional chemical functionality in the bifunctional ligand can influence its pharmacokinetics and this can be advantageous. Thus, studies of an analogous Dex conjugate series indicate that the thiourea linker used here confers favorable properties both for cellular permeability (44) and transscleral transport in the context of ocular delivery (45). Our data also indicate that the FK506 moiety enhances the cellular potency of Dex (Figure 4) as well as RU486-based bifunctional ligands (Figure 6) relative to their intrinsic receptor affinities (Figure 3), an effect observed even in the absence of designed FKBP fusion proteins (Figure 6B, left panel). The enhanced potency may be a reflection of increased cellular uptake or retention provided by the FK506 group, particularly because FK506 can serve as a substrate and inhibitor of drug efflux transporters such as multidrug resistance protein 1 (41). Notably, the favorable pharmacokinetic properties provided by FK506 have been recently demonstrated for drug conjugates both in vitro and in vivo (42, 43). Similarly, conjugation of GR antagonists to bile acids has been explored as a means to target GR antagonism to the liver (28). The oral bioavailability, tissue distribution, and microsomal stability of such conjugates (28) indicate that GR bifunctional ligands can have pharmacokinetic properties suitable for further clinical development.

The experiments presented here depend on genetic manipulation of the designed coregulator. Although this may be incorporated as part of gene therapy strategies, transition to bifunctional ligands acting on purely endogenous proteins will obviously depend on appropriate functionalities that can target coregulator complexes. In this regard, the recent progress by our group (46–48) and others (49) in the development of small molecules that can

mimic activation domains offers some clear opportunities (50). Enhancing the agonist efficacy of glucocorticoid ligands in this manner could provide a means to overcome or alleviate steroid resistance, which is an important clinical problem in diseases such as asthma (51, 52), nephrotic syndrome (53), and malignancies such as acute lymphoblastic leukemia (54). Similarly, such ligands could restore function to carriers of mutations in GR that impair interaction with coactivators (55, 56). Indeed, the modular design of our bifunctional ligands argues for the successful incorporation of these chemical motifs to target endogenous regulatory complexes.

Bifunctional ligands made as FK506 conjugates as in this study have intriguing prospects in their own right as mechanistic tools. They can be used to directly recruit specific FKBP-coactivator or -corepressor fusions. This can provide a means to not only identify factors that can overcome gene-specific, rate-limiting barriers to activation but also to provide novel mechanisms of repression for specific GR target genes. For example, extrinsic recruitment of factors implicated in the GR-mediated repression at tethering sites such as negative elongation factor (14) could enhance the repressive effects of agonists in a gene-selective manner. By examining multiple cofactors in parallel, this approach could also be used to establish epistatic relationships between them and the gene subsets affected by them. It is precisely this type of knowledge that is required to identify the most desirable regulatory profile for a given therapeutic application. Furthermore, the approach could also be used to establish or monitor specific epigenetic marks at GR-targeted loci in a ligand-dependent manner as has been recently illustrated for octamer-binding transcription factor 4 (57). Clearly the strategy outlined here has significant potential and opens up the possibility of an instructive ligand design not only for GR but also for the entire nuclear receptor class.

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APPENDIX II: ABSTRACTS OF PRESENTATIONS

Abstract for the Vaughn Symposium 2012, July 30, 2012, University of Michigan
Authors: Steven Sturlis (presenting author), James Carolan, Aaron van Dyke and Anna K Mapp

According to the American Cancer Society, prostate cancer is the second leading cause of cancer related deaths in American men and one in six men will be diagnosed at some point in their lifetimes. Aberrant activation of androgen receptor (AR), a ligand-regulated transcription factor in the nuclear receptor subclass of proteins, has been strongly implicated in prostate cancer pathogenesis. In the early stages of the disease, androgen ablation therapy is utilized to suppress AR function by limiting the production of endogenous androgens. In the majority of cases, the disease eventually progresses to a hormone refractory state, in which AR is required for cancer cell survival and continues to function in the absence of endogenous androgens through a variety of mechanisms. Hormone refractory prostate cancer is a terminal illness, underscoring the need for the development of novel therapeutic strategies.

To this end, a potent androgen receptor agonist has been synthesized, to which a polyethylene glycol (PEG) linker has been appended. Early biological characterization using chromatin immunoprecipitation has been carried out, demonstrating the ability of the modified ligand to effectively localize and bind AR to DNA. Additionally, a histone deacetylase inhibitor is being investigated for potential use within the proposed strategy.

Abstract for the Novartis Symposium 2012, October 15, 2012, University of Michigan
Authors: Steven Sturlis (presenting author), James Carolan, Aaron van Dyke and Anna K Mapp

Title: Bifunctional Small Molecules Targeting the Androgen Receptor
Department: Chemistry
Abstract:

Aberrant activation of the androgen receptor (AR), a member of the nuclear receptor superfamily, has been strongly implicated in the pathogenesis of prostate cancer, which is the second leading cause of cancer related deaths in men according to the American Cancer Society. The disease is initially treated through androgen ablation therapy to reduce the production of endogenous ligands for the receptor. However, in the majority of cases, the disease progresses to a hormone refractory state, in which AR function is restored, despite low androgen concentrations. At this stage, the disease becomes terminal, underscoring the need to develop novel therapeutic strategies.

One potential strategy to suppress AR regulated gene expression is to recruit corepressor complexes that contain histone deacetylase (HDAC) activity to the promoters of AR controlled genes. HDACs repress gene expression by deacetylating core histones, which results in the tighter compaction of chromatin and reduces the ability of transcription factors to bind. It is hypothesized that the recruitment of these complexes to AR regulated promoters will lead to the downregulation of genes required for prostate cancer cell survival. Bifunctional molecules capable of binding

both AR and HDAC complexes present an attractive approach to implementing this strategy and are currently being investigated.

To this end, a potent AR ligand has been synthesized, to which a polyethylene glycol linker has been appended. Early biological characterization using chromatin immunoprecipitation has demonstrated the ability of the modified ligand to effectively localize and bind AR to DNA. Additionally, an HDAC inhibitor is currently being investigated for potential use within the proposed strategy.

Presented by Dr. Aaron van Dyke at the 2013 Bioorganic Gordon Research Conference and the 2013 Regional ACS meeting *Dr. van Dyke recently (Sept 2013) assumed an independent position at Fairfield University.*

Title: Modulating Gene Expression with Bifunctional Ligands

Authors: Aaron Van Dyke, Jun Qi, Jonas Hojfeldt, Osvaldo Cruz, Jorge Iniguez-Lluhi, James Bradner, Anna Mapp

Abstract:

Glucocorticoid receptor (GR) is a ligand-inducible transcription factor that regulates gene expression by recruiting protein cofactors to DNA. Errors in this process correlate with a range of human cancers, making GR an important therapeutic target. Historically, GR has been modulated by small molecule ligands that allosterically recruit cofactors. Alternatively, bifunctional ligands that can simultaneously bind GR and cofactor would be powerful tools to directly recruit proteins to DNA. By directly controlling cofactor recruitment to DNA, we aim to achieve greater control over gene expression. As a proof of principle, a collection of bifunctional molecules were synthesized to recruit the artificial activator VP16 to GR. VP16 recruitment activates gene transcription to higher levels than could previously be achieved with classical GR ligands. Current efforts to recruit naturally occurring coactivators in living cells will also be discussed.

Presented by graduate students Steven Sturlis and James P. Carolan at the 2013 Novartis Symposium

Title: Bifunctional Small Molecules Targeting the Androgen Receptor

Abstract:

Aberrant activation of the androgen receptor (AR), a member of the nuclear receptor superfamily, has been strongly implicated in the pathogenesis of prostate cancer, which is the second leading cause of cancer related deaths in men according to the American Cancer Society. The disease is initially treated through androgen ablation therapy to reduce the production of endogenous ligands for the receptor. However, in the majority of cases, the disease progresses to a hormone refractory state, in which AR function is restored, despite low androgen concentrations. At this stage, the disease becomes terminal, underscoring the need to develop novel therapeutic strategies.

One potential strategy to suppress AR regulated gene expression is to recruit corepressor complexes that contain histone deacetylase (HDAC) activity to the promoters of AR controlled genes. HDACs repress gene expression by deacetylating core histones, which results in the tighter compaction of chromatin and reduces the ability of transcription factors to bind. It is hypothesized that the recruitment of these complexes to AR regulated promoters will lead to the downregulation of genes required for prostate cancer cell survival. Bifunctional molecules capable of binding both AR and HDAC complexes present an attractive approach to implementing this strategy and are currently being investigated.

To this end, a potent AR ligand has been synthesized, to which a polyethylene glycol linker has been appended. Early biological characterization using chromatin immunoprecipitation has demonstrated the ability of the modified ligand to effectively localize and bind AR to DNA. Additionally, an HDAC inhibitor is currently being investigated for potential use within the proposed strategy.

November, 2014 - Life Sciences Institute Annual Poster Session (Univ of Michigan, Ann Arbor, MI) - title: Alternative Modulation of Glucocorticoid Receptor Activity

Abstract: Nuclear receptors (NRs) such as the glucocorticoid receptor (GR) are ligand-inducible transcription factors that regulate gene expression by recruiting protein complexes to DNA. Misregulation of this process has been implicated in a range of disease states. Historically, NRs have been therapeutically modulated by small molecules that *allosterically* recruit coregulator complexes that up- or down-regulate gene expression. Alternatively, bifunctional molecules that *directly* control coregulator recruitment would be excellent tools to provide new profiles of gene expression. Bifunctional molecules that target the GR and the lysine reader protein BRD4 have been synthesized and are being evaluated for activity *in vitro*, with the results being discussed here.

August, 2014 - 249th ACS National Meeting and Exposition (San Francisco, CA) - title: Alternative Modulation of Glucocorticoid Receptor Activity Using Bifunctional Small Molecules

Abstract: Nuclear receptors (NRs) such as the glucocorticoid receptor (GR) are ligand-inducible transcription factors that regulate gene expression by recruiting protein complexes to DNA. Misregulation of this process has been implicated in a range of disease states. Historically, NRs have been therapeutically modulated by small molecules that *allosterically* recruit coregulator complexes that up- or down-regulate gene expression. Alternatively, bifunctional molecules that *directly* control coregulator recruitment would be excellent tools to enhance control of gene expression. Bifunctional molecules that target the GR and the lysine reader protein BRD4 have been synthesized and are being evaluated for activity *in vitro*, with the results being discussed here.

July, 2014 - Victor Vaughan Symposium (Univ of Mich, Ann Arbor, MI) title:

Alternative Modulation of Glucocorticoid Receptor Activity Using Bifunctional Small Molecules

Abstract: Nuclear receptors (NRs) such as the glucocorticoid receptor (GR) are ligand-inducible transcription factors that regulate gene expression by recruiting protein complexes to DNA. Misregulation of this process has been implicated in a range of disease states. Historically, NRs have been therapeutically modulated by small molecules that *allosterically* recruit coregulator complexes that up- or down-regulate gene expression. Alternatively, bifunctional molecules that *directly* control coregulator recruitment would be excellent tools to enhance control of gene expression. Bifunctional molecules that target the GR and the lysine reader protein BRD4 have been synthesized and are being evaluated for activity *in vitro*, with the results being discussed here.